AD_____

GRANT NUMBER DAMD17-96-1-6052

TITLE: Isolation of Breast Tumor Suppressor Genes from Chromosome 11p

PRINCIPAL INVESTIGATOR: Doctor Pratima Karnik

CONTRACTING ORGANIZATION: Cleveland Clinic Foundation
Cleveland, OH 44195

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (23 Aug 97 - 22 Aug 98)

4. TITLE AND SUBTITLE Isolation of Breast Tumor Supressor Genes from Chromosome 11p

5. FUNDING NUMBERS
DAMD17-96-1-6052

6. AUTHOR(S)
Dr. Pratima Karnik

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cleveland Clinic Foundation
Cleveland OH 44195

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

See Page 5 for Abstract

14. SUBJECT TERMS Breast Cancer

15. NUMBER OF PAGES
55

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

PK ✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

PK ✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PK ✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

PK ✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Pratima Karnik Sep. 22 1998

PI - Signature Date

TABLE OF CONTENTS:

ABSTRACT

Genetic alterations of the short arm of chromosome 11 are a frequent event in the etiology of cancer. Several childhood and adult tumors demonstrate LOH for 11p suggesting the presence of one or more critical tumor suppressor gene(s) involved in several malignancies. We have previously described two regions of frequent allelic loss on the short arm of chromosome 11 in sporadic breast cancer that are likely to harbor tumor suppressor genes. The first interval is bordered by the markers D11S1318 on the telomeric side and - D11S4088 on the centromeric side of chromosome 11p15.5. A second, critical region of LOH spans the markers D11S1338-D11S1323 at 11p15.5-p15.4. There is a striking correlation between the loss of the two 11p loci and the clinical and histopathological features of breast tumors suggesting that the target genes may contribute to tumor progression and metastasis in breast cancer. Using a combination of EST mapping and gene prediction methods like GRAIL and PowerBlast, we have established transcript maps of LOH regions 1 and 2. We have identified nine novel Unigenes in region 1 and are assessing these genes as candidate tumor suppressors by Northern analysis of normal tissue, normal/tumor breast tissue and breast cancer cell lines. In addition, we are screening tumor samples for mutations by SSCP. We have cloned and characterized a novel gene, *HET* (Human Efflux Transporter), that is widely expressed in adult and fetal tissues and has homology to known drug-efflux pumps and multi-drug resistance proteins in bacteria. Most fetal tissues show monoallelic expression, suggesting that *HET* is imprinted. Thus, dosage regulation of the efflux transporter might control fetal growth and altered imprinting could potentially contribute to pathogenesis. Several other novel genes are of particular interest and are currently being assessed as putative tumor suppressors.

INTRODUCTION

Breast cancer is both genetically and clinically a heterogeneous and progressive disease. The severity of disease may be determined by the accumulation of alterations in multiple genes that regulate cell growth and proliferation. The inactivation of tumor-suppressor genes, by a 2-hit mechanism involving mutations and loss of heterozygosity (LOH), appears to be a common event in the genetic evolution of breast carcinomas (1). Several chromosome arms, including 1p, 1q, 3p, 11p, 11q, 13q, 16q, 17p, 17q and 18q have been reported to show moderate (20-40%) to high (>50%) frequencies of LOH in breast tumors (1). This implies that multiple tumor suppressor genes are likely involved in the development and progression of breast cancer.

Genetic alterations at the short arm of chromosome 11 are a frequent event in the etiology of cancer (2-16). Several childhood tumors demonstrate LOH for 11p including rhabdomyosarcoma (7), adrenocortical carcinoma (8), hepatoblastoma (9), mesoblastic nephroma (10) and Wilms' tumors (11). Recurrent LOH at 11p is also observed in adult tumors including bladder (12), ovarian (13), lung carcinomas (14), testicular cancers (15), hepatocellular carcinomas (16) and breast carcinomas (2-6) suggesting the presence of one or more critical tumor suppressor gene(s) involved in several malignancies.

Birch et. al. (17) have reported an increased risk for breast cancer among mothers of children with embryonal rhabdomyosarcoma, providing genetic evidence for the apparent high-risk association between these two tumor types. The familial association between breast cancer and rhabdomyosarcoma and the other childhood tumors may well be the consequence of alterations in chromosome 11p15. The ability of a tumor suppressor gene(s) on chromosome 11 to re-establish control on the malignant phenotype has been demonstrated by transfer of a normal human chromosome 11 to the breast cancer cell line MDA-MB-435 (18). However, positional cloning efforts to identify the target genes on 11p15 have been complicated by the large size of this region (~10 Mb) and complexity of LOH at 11p15.

BODY

A. Results and Discussion:

We have recently identified and fine-mapped two distinct regions on chromosome 11p15.5 that are subject to LOH in breast (19) and Wilms tumors (20). The telomeric region extends between the markers D11S1318 and D11S1288 and the more centromeric region extends between the markers D11S1338 and D11S1323. LOH analysis of 94 matched normal and breast tumor samples using 17 polymorphic markers that map to 11p15.5-15.4 has enabled us to precisely define the location of a breast tumor suppressor gene between the markers D11S1318-D11S4088 (~460 kb) within 11p15.5. LOH at this region occurred in ~35-45% of breast tumors analyzed.

In addition, we have fine-mapped a second, critical region of LOH that spans the markers D11S1338-D11S1323 (~336 kb) at 11p15.5-p15.4, that is lost in ~ 55-60% of breast tumors. There is a striking correlation between the loss of the two 11p loci and the clinical and histopathological features of breast tumors. LOH at region 1 correlated significantly ($p = 0.016$) with early events in malignancy and invasiveness. In contrast, the loss of the more proximal region 2, is highly predictive ($p = 0.012$) of aggressive metastatic disease. Thus, two distinct tumor suppressor loci on chromosome 11p15 may contribute to tumor progression and metastasis in breast cancer. The fine mapping of this intriguing chromosomal region should facilitate the cloning of the target genes and provide critical clues to understanding the mechanisms that contribute to the evolution of adult and childhood cancers. The characterization of novel genes that map to this region should provide evidence for or against their involvement in tumor development and enhance our understanding of the role this chromosomal region plays in tumorigenesis. The construction of a detailed transcription map of the 11p15 LOH regions is an essential step toward the identification of specific genes important to the etiology of several childhood and adult tumors including breast cancer.

A1. Transcript map of LOH Region 1:

The LOH region 1 spans a distance of ~ 460 kb between the markers D11S1318 and D11S4088. The large-scale genomic sequencing of chromosome 11p15 has been an ongoing effort as part of the Human Genome Project (http://mcdermott.swmed.edu). The PAC, BAC and P1 clones that overlap the D11S1318-D11S4088 LOH region (pDJ1075F20, pDJ915f1, pDJ608B4, BAC70i6 and PAC 74k15 -See Figure 1) have been sequenced. We have used GRAIL (21) and PowerBlast (22) on the genomic sequences to establish a transcript map of the proposed critical region between the markers D11S4088 and D11S1318 (Figure 1) This approach has allowed the identification of three known genes that were previously mapped to this region (*ASCL2, TAPA1 and KVLQT1*).

PowerBlast identified several additional ESTs that did not correspond to any known previously identified genes (Figure 1). EST clusters that overlapped apparently transcribed Alu or L1 repeat sequences were also identified. The EST's were queried against the Unigene database to identify homologous gene sequences. Using this approach, nine predominant EST clusters were identified in LOH region 1 (Fig. 1). Such EST clusters can represent novel transcripts, often referred to as "Unigenes" (23). Unigene cluster 1 mapped between the TH and the ASCL2 genes and contained ESTs expressed in breast, kidney and fetal tissues. Unigene clusters 2 and 3 mapped between the D11S1318 marker and the TAPA1 gene. While the unigene cluster 3 contained ESTs expressed only in fetal tissues, unigene cluster 2 contained ESTs expressed in breast, kidney, lung and fetal tissues. Five novel unigene clusters were identified between the TAPA1 and the KVLQT1 genes. As described in Fig.1, these clusters contained ESTs expressed in tissues like breast, kidney and skeletal muscle. This is significant because tumors that arise in these tissues show a high frequency of LOH at 11p15.5. A single unigene cluster was identified between the KVLQT1 gene and D11S4088 marker. This cluster was also expressed in all important tissues. Figure 2shows the multiple tissue Northern blots hybridized to a

representative cDNA clone from several different Unigene clusters. With the goal of identifying the target tumor suppressor gene, we are currently determining the expression patterns of these ESTs and performing mutation analysis by PCR-SSCP in normal breast and corresponding tumor tissues.

A2. Monoallelic expression of the gene encoding a Human Efflux Transporter like protein (HET), on chromosome 11p15.5:

In our continuing efforts to identify the candidate tumor suppressor genes on chromosome 11p15.5, we have cloned and characterized a novel gene, *HET* (Human Efflux Transporter), that is widely expressed in adult and fetal tissues. *HET* is located ~ 3 kb telomeric to *IPL* and ~16 kb centromeric to p57KIP2. The 1.5 kb transcript encodes a predicted protein of 408 amino acids with a molecular weight ~ 43 kDa. We have defined the intron-exon structure of *HET* and characterized a number of splicing variants of *HET* mRNA that could give rise to sequence diversity in the protein. The secondary structure of the *HET* protein displays ten putative helical transmembrane domains, a structural feature shared by all members of integral membrane transporter proteins. Furthermore, the homology to known drug-efflux pumps in bacteria raise the possibility that this gene may play a role in organic solute transport or drug elimination. Most fetal tissues show monoallelic expression, suggesting that *HET* is imprinted. Thus, dosage regulation of the efflux transporter might control fetal growth, and altered imprinting could potentially contribute to tumorigenesis.

A2a. Cloning and Characterization of the HET gene:

The unigene cluster proximal to the KVLQT1 gene (Fig. 1) contains 14 overlapping ESTs. One of the ESTs from this cluster was used to screen a fetal kidney cDNA library (Clontech), and several clones ranging in size from 600 kb to 1.5 kb were isolated and sequenced. The 1.5 kb cDNA clone was found to contain an open reading frame, with a

putative initiator methionine at position 243, and an inframe downstream stop signal, encoding a 408 amino acid polypeptide having a calculated relative molecular weight of approximately 43 kDa (Figure 3B). A distal inframe ATG, which is in a poorer initiation context is also present at position 195. The 3'- untranslated region contains the hexanucleotide 5'- AATAAA-3', in the expected position, 23 nucleotides downstream from the termination codon. The novel gene maps ~ 3 kb distal to *IPL* and ~16 kb proximal to *p57KIP2* (Figure 3A). The predicted protein harbors similarities and has ~21-23% homology to the bacterial energy dependent efflux pumps that transport a variety of toxic substances out of the cell (23,24). A common feature of all these proteins is that they are integral membrane proteins with multiple trans-membrane domains.

The overall deduced HET protein is very hydrophobic. The hydrophobicity profile of HET (Figure 4) according to the Kyte-Dolittle algoritm (26) shows the presence of ten strong trans-membrane helices with the N- and C- termini facing the cytoplasmic side. The protein contains thirteen potential N-myristoylation sites and one putative prenylation site. Furthermore, there are two potential protein kinase C and three casein kinase II phosphorylation sites.

The tissue specific expression of *HET* was determined by Northern blot hybridization using a full-length cDNA probe at high stringency. The results of the hybridization (Figure 5), revealed two primary transcripts of 4.4 kb and 1.5 kb in most human tissues. The 4.4 kb or 1.5 kb transcripts, alone or together, were expressed in different human cancer cell lines (Figure 5) from promyelocytic leukemia, (HL-60), lymphoblastic leukemia (MOLT-4), Burkitt lymphoma (Raji), lung carcinoma (A549), and chronic myelogeneous leukemia (K-562).

A2b. Characterization and expression of alternatively spliced variants of HET:

Alignment of the cDNA sequence of *HET* with the genomic sequence for the region of chromosome 11p15.5 containing *HET* (244 kb contig; accession no.U90582) allowed the position of intron/exon boundaries to be predicted. The *HET* gene spans approximately 25 kb, and the open reading frame is contained in 11 exons (Figure 6a), with the direction of transcription being from telomere to centromere. The intron sizes range from 220 (intron 4) to 6908 bp (intron 5) (Table 1). All exon/intron splice junctions conform to the eukaryotic 5' donor 3' acceptor consensus splice junction GT/AG rule (27). Junctions at introns 2, 8 and 9 are type 0 (splicing occurring between codons), junctions at introns 4, 6, 7 and 10 are type 1 (splicing occurring after the first base of the codon), and the junction for introns 3 and 5 are type 2 (splicing occurring after the second base of the codon). The full length clone of 1.5 kb, represented as isoform 1 contains exons 1 through 11, with the ATG start codon in exon 2 and a TGA stop codon in exon 11 (Figure 6a). Three additional cDNA clones isolated from a kidney cDNA library were sequenced and found to be isoforms of *HET*. Isoform 2 lacks exon 1 and contains a new exon at the 5'end designated as exon 1a, indicating alternate splicing at the 5'end of *HET*. The 1.5 kb cDNA clone for isoform 2 contains the same open reading frame as isoform 1.

Two additional alternatively spliced cDNAs, designated isoforms 3 and 4 (Figure 6a) contain new exons that introduce stop codons into the *HET* sequence, suggesting that isoforms 3 and 4 represent untranslated transcripts. The 1.2 kb cDNA clone for Isoform 3 contains a new exon 5a, between exons 5 and 6, and lacks exons 1 through 5. The Isoform 4 cDNA clone (900 bp), contains a new exon 6a, between exons 6 and 7, and lacks exons 1 through 6. Figure 6b shows the sequence of the novel exons 1a, 5a and 6a. All the isoforms contain exons 7 through 11, a polyadenylation signal and poly (A) tails at their 3' ends. However, the 5' ends of the isoforms are different because

of different exons that are utilized, suggesting alternative splicing of *HET*. We have not excluded the presence of additional 5' exons in isoforms 3 and 4, but an attempt at 5' extension by PCR of total adapter-ligated kidney cDNAs (5' RACE) did not yield clones extending beyond exons 5a and 6a in the two alternatively spliced isoforms.

Because *HET* exhibits complex alternative splicing, we sought to determine whether any isoforms show tissue-specific expression. Figure 6c shows the expression pattern of the four isoforms in different human tissues as determined by RT-PCR. Isoform 1 is expressed in all tissues tested , while isoform 2 is expressed only in bone marrow and fetal kidney. Isoforms 3 and 4 are expressed at much lower levels in the fetal kidney.

A2c. Identification of a transcribed polymorphism and monoallelic expression of HET:

Single strand conformational analysis (SSCA) (28) of the human *HET* gene revealed a transcribed polymorphism in exon 2. Figure 7a shows the location of the polymporphism and the primers used for the SSCA analysis. Using the expressed polymorphism, we investigated LOH at this locus in informative breast and Wilms tumors. LOH was observed in 4/10 breast tumors (data not shown) and 3/10 Wilms tumors. Figure 7b shows the SSCA pattern of exon 2 amplified from paired normal/tumor samples from four different Wilms tumor patients. Sample 1 is homozygous, while the remaining three samples are heterozygous with the tumors showing loss of heterozygosity (LOH). LOH was confirmed using flanking microsatellite markers D11S1288 and D11S4088, which are located centromeric and telomeric to HET, respectively (data not shown). The presence of only one allele in the DNA of the tumor confirmed that the two bands observed on SSCA-PCR of normal tissue correspond to the two alleles of the gene.

SSCA of samples shown in Figure 7b along with other samples analyzed (data not shown), revealed the presence of five variant alleles. The sequence of the polymorphism was confirmed by direct sequencing (Figure 7c), which revealed heterozygosity in the

normal DNA of sample 45 and monoallelic representation in 45T that shows LOH. The human *HET* polymorphisms are G→A or A→G transitions at nucleotide positions 207 and 226 respectively. These polymorphisms reside outside of the potential ORF at position 243. The five variant alleles are produced by different combinations of these two polymorphisms. Both polymorphisms are easily detected by RT-PCR SSCP.

Parallel genomic and cDNA PCR (RT-PCR) of exon 2, followed by SSCA analysis revealed heterozygosity in two of the five fetuses (Figure 8). Comparison of the SSCA band patterns of genomic and cDNA PCR products (Figure 8a and 8b) suggested monoallelic expression of *HET* with one individual showing only the 'upper' allele/SSCA conformer in the cDNA lane and the other expressing only the 'lower' allele/SSCA conformer. In cases where more than one tissue was available, all tissues showed the same allelic expression bias, an observation that is consistent with imprinting. We are currently investigating the parental origin of the functionally imprinted *HET* allele and its variant isoforms. The *HET* gene maps within the imprinted cluster of five human genes, on the chromosomal band 11p15.5, in the order cen-IPL-HET-p57KIP2-KVLQT1-IGF2-H19-tel.

A2d. Mutation analysis of the HET gene:

The map location of *HET* makes it a potential tumor suppressor candidate gene. To assess whether *HET* contributes to breast cancer or Wilms tumor, we analyzed DNA from 40 breast tumors and 35 Wilms tumors using SSCA analysis on the predictive protein coding exons of the *HET* gene and its isoforms. Mutations were not detected since SSCA shifts were observed both in normal and tumor DNAs and therefore represent sequence polymorphisms. The focus of our current studies is to determine if alterations in imprinting of *HET* could contribute to tumorigenesis.

A3. TRANSCRIPT MAP OF LOH REGION 2:

A3a. Genomic Contig:

The LOH region 2 extends between the markers D11S1338-D11S1323 on chromosomal band 11p15.4-p15.4. Since the genomic sequence of this region has not yet been determined, we established a genomic contig across this region (Figure 9). The YAC contig (clones 847a12 and 696H10) was generated with CEPH maga-YACs obtained from CEPG-Genethon and MIT databases. The BAC 1323 clone was screened from a human bacterial artificial chromosome (BAC) library release III obtained from Research Genetics. The PAC clone (PACILK) was isolated from a PAD10SacBII library (29). The cosmids 141b9, 173c9 and 49h3 were identified by colony hybridization of a human cosmid library prepared from normal lymphocyte DNA.

A3b. EST mapping:

The development of expressed sequence tags (ESTs) has greatly facilitated the generation of a human gene transcript map (23). ESTs, short stretches of sequences generated from cDNA clones, can be easily converted to a PCR format and assigned chromosome locations using genomic contigs. We tested 96 ESTs derived from unidentified transcripts mapped to region 11p15.5-p15.3 within an interval defined by loci D11S909 at the centromeric side and D11S1318 at the telomeric side (23). As shown in Figure 9, sixteen of the 96 ESTs mapped to our genomic contig that spans ~ 1Mb. The smallest common deleted region (SCDR) as determined from our LOH studies extends between the markers D11S1338 and D11S1323 (19), a distance of ~ 336 kb as determined from this contig.

To search for homologies, a BLAST search of the mapped ESTs against non-redundant sequences in Genbank was carried out. Of the 16 ESTs that map to our contig,

Figure 9 and Table 2), SGC30555 has strong homology to the 60S ribosomal protein L21 and SGC33125 has strong homology to the 50S ribosomal protein L17. The EST SGC35504 was identified as the inteferon inducible gene Staf 50 (30) and WI6973 was identified as the sphingomyelin phosphodiesterase gene SMPD1. The nucleotide sequences of the remaining 12 ESTs revealed no homology with any known gene.

In addition to the 16 ESTs, we have mapped two previously identified genes namely Integrin -linked kinase (p59ILK) (31) and the gene encoding the human TBP-Associated Factor II 30 ($TAF_{II}30$) (32) to our genomic contig. p59ILK was previously mapped to the CALC-HBBC region on chromosome 11p15 (31). We have refined the map location of p59ILK and $TAF_{II}30$ placed it on a yeast artificial chromosome (YAC) 847a12 that is 1440 kb and contains the markers D11S1338 and D11S1323. PCR amplification of DNA from the YAC 847a12 with several different p59ILK and $TAF_{II}30$ primers produced the expected length fragments (Figure 10). No PCR products were seen from the BAC DNA specific for the marker D11S1323 or from yeast DNA. ILK is a serine threonine protein kinase that is thought to be involved in signal transduction by ß1 family integrins. ILK has been shown to induce anchorage-independent growth in a rat epithelial cell line (31). This data combined with our observation of a strong correlation between LOH in region 2 and metastatic phenotype of breast tumors (19), makes ILK a very strong candidate tumor suppressor gene for LOH region 2. TAFs are involved in transcriptional initiation and activation, and it remains to be determined whether TAF_{II} 30 dysfunction could be involved in tumorigenesis ..

The refined subchromosomal EST and gene mapping contributed by the present work provide valuable candidate genes that should facilitate the identification of the target gene in this region.

B. Materials and Methods:

B1. Molecular cloning and Sequence Analysis:

The genomic sequence of the 244 kb contig from human chromosome 11p15.5 spanning D11S1 through D11S25 (GenBank accession no.U90582) was generated at the McDermott Center at the University of Southwestern (http://mcdermott.swmed.edu). Genomic DNA was assessed for coding potential using the client program PowerBLAST. PowerBlast software was obtained from the National Center for Biotechnology information (ftp:ncbi.nlm.nih.govcd pub/sim2/PowerBlast). Database and sequence analyses were performed using a network version of GRAIL (http://avalon.emp.ornl.gov/GRAIL-bin/emptygrailform) and BLAST network service from the National Center for Biotechnology Information. Transmembrane alpha helix prediction was performed using the Protean program from the DNASTAR™ software package. A combined strategy of EST database searches and direct screening of a fetal kidney cDNA library (Clontech, PaloAlto, CA) was used to obtain the human *HET* cDNA sequence and its various isoforms. Plasmid DNA was sequenced with vector or insert specific primers (see below), fluorescence-tagged dinucleotides, and the TAQ cycle sequencing procedure (ABI). PCR products were purified with Micron-100 microconcentrators (Amicon). Sequencing reactions were analyzed on an ABI DNA sequencer 373A. The I.M.A.G.E Consortium (LLNL) cDNA clone AA573743 (http://www-bio.llnl.gov/bbrp/image/image.html/) was purchased from Research Genetics. Tissue-specific expression was analyzed using multiple tissue Northern blots (Clontech) following standard methods (33).

B2. PCR, RT-PCR and SSCP analysis:

DNA was extracted from the tissues as previously described (34). Total RNA was prepared by extracted with Trizol-reagent (GIBCO-BRL) and in some cases subjected to mRNA enrichment by oligo-dT chromatography. The *HET* sequence polymorphism was

detected by PCR with the two intronic primers A6 and B6 (A6: 5'-GATCAACTGGACTTTTGCCC-3', B6:5'GGAGAACTGCATGAAGAGGC-3') (Figure 7). Cycling conditions were an initial denaturation for 5 min at 95 °C followed by 30 cycles of annealing at 60° C for 1 min, extension at 72 ° C for 1 min and denaturation at 95 °C for 1 min, and a final extension at 72 ° C for 5 min. RT-PCR was performed on total RNA or poly (A) RNA isolated from the tissues of heterozygous individuals. cDNA was synthesized from approximately 1μg of total RNA or 200 ngs of poly (A) RNA using Mulv reverse transcriptase (Perkin Elmer). PCR reactions contained 0.5 μM primers IP1 and IP2. (IP1: 5'CAGTGACTCAGCACCCCTG3';
IP2: 5'GAAGTTGGTTTGCAGGTAGC3'), 0.2mM dNTP, 50ng cDNA, 1X PCR buffer, 1μCi[α-^{32}P] dATP, and 0.5U *Taq* DNA polymerase (BRL) in 25μl and were performed with an Ericomp thermocycler as follows: 30 cycles of 94°C for 1min, 60°C for 1min and 72°C for 1min, followed by primer extension at 72°C for 10 min. For SSCA analysis, the PCR products were denatured at 94°C for 4 min. and analyzed by 6% polyacrylamide gel electrophoresis at 8W at room temperature overnight as described earlier (28). Tissue specific expression of the various isoforms was determined by RT-PCR with alternate exon-specific primers and analyzed by ethidium bromide stained agarose gels. The primers used to identify the different isoforms were as follows: Isoform 1 (primers IP1 and IP2), isoform 2 (GL5-5'TCACGTCCTGTCATTCTTGC3'; and GL6-5'GTGGTGTACAGCGTTCCTGA3'), isoform 3 (GL1: 5'CTGGCATTTTCCCAAAGGAA3'; and GL2: 5'CAGAGCAAGGAGGGGACTTG3') isoform 4 (GL3: 5'TGCGTAGAAGTGTGCTGAGG3'; GL4: 5'AACATGACCATGAAGAGCCC3').

B3. EST Primers:

Oligonucleotide primers were obtained from Research Genetics (Huntsville, Ala.). ESTs were chosen on the basis of their mapping on he human genome. Information was obtained from the world wide web page for chromosome 11 found at www.ncbi.nlm.nih.gov/SCIENCE96/. Polymerase chain reactions were carried out using 50 ngs of YAC, BAC, PAC or cosmid DNA at the following conditions: 35 cycles of a denaturation step at 94°C 15s and extension step at 72°C 30s.

B4. Patient Materials and Preparation of Genomic DNA :

Primary tumor and adjacent normal breast tissue samples were obtained from 94 randomly selected breast cancer patients undergoing mastectomy at the Cleveland Clinic Foundation (CCF). Samples of these tumors and corresponding noninvolved tissue from each patient were collected at the time of surgery, snap-frozen, and transferred to -80°C. An initial cryostat section was stained with H and E stain to determine the proportion of contaminating normal tissue and only DNA purified from specimens thought to be highly enriched in tumor tissue was used for PCR. Generally we use tumor samples that contain less than 40% contamination of normal cells. Genomic DNA was isolated from normal and tumor tissue samples as described earlier and quantitated by determining the optical densities at 260 and 280 nm.

CONCLUSIONS

Genetic loci responsible for human diseases and malignancies are constantly being assigned to chromosome regions using a variety of methods including linkage analysis, associations with chromosome rearrangements and loss of heterozygosity studies (LOH). Once their chromosomal localization has been established, it is then usually necessary to

refine the the localization to isolate it. In many cases this involves the construction of contiguous arrays (contigs) of overlapping DNA clones. Despite recent advances in methodology for isolating transcribed sequences from these contigs, this is still a very time-consuming and complicated process. The large scale sequencing of human chromosomes as part of the Human Genome Project, has greatly facilitated the identification of transcribed sequences using computer-based techniques like GRAIL and POWERBLAST. We find that PowerBlast analysis of dbEST is the most useful single tool to apply to the genomic sequence for identifying novel genes. Using this approach we have discovered a new gene and confirmed that it is expressed in a wide range of tissues by RT-PCR and Northern blot analysis. The gene designated as Human Efflux transporter (*HET),* encodes a predicted protein of 408 amino acids with a M_r of 43kDa, and maps ~ 3kb distal to the IPL gene and ~ 16 kb proximal to the p57 KIP 2 gene. Blastp analysis of the predicted *HET* protein identified several proteins belonging to the family of multi-drug efflux transporters in bacteria .

An alternative approach to identifying the gene responsible for the phenotype is through the evaluation of potential candidate genes that map to the same region of the chromosome. The major problem with this approach at the moment is the paucity of genes that have already been assigned not only to individual chromosomes but, more importantly, to subregions of the chromosome . The recent publication of the transcript map of the whole human genome (23) provides the framework to better define, with the help of YAC contigs, the regions where the ESTs have been broadly defined. This approach in turn provides a series of genic markers in which to search for candidates for a variety of genetic disorders. We have used successfully, both the above mentioned approaches, to establish transcript maps of the two LOH regions on chromosome 11p15. This should greatly speed the isolation of genes linked to the disease loci.

RECOMMENDATIONS IN RELATION TO THE STATEMENT OF WORK OUTLINED IN THE PROPOSAL:

We have completed Aims 1 and 2 of our Statement of Work described in the research proposal. This work is currently being prepared for publication. The work is progressing on schedule and meets our expectations.

REFERENCES:

1. Callahan, R and Campbell, G.N. Mutations in human breast cancer: an overview. J. Natl. Cancer Inst. *81*: 1780-1786, 1989.

2. Ali, I.U., Lidereau, R., Theillet, C. and Callahan, R. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science *238*: 185-188, 1987.

3. Winquist, R., Mannermaa, A., Alavaikko, M., Blanco, G., Taskinen, P.J., Kiviniemi, H., Newsham, I. and Cavenee, W. Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. Cancer Res. *55*: 2660-2664, 1995.

4. Negrini, M., Rasio, D., Hampton, G.M., Sabbioni, S., Rattan, S., Carter, S.L., Rosenberg, A.L., Schwartz, G.F., Shiloh, Y., Cavenee, W.K. and Croce, C. Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: Identification of a new region at 11q23.3. Cancer Research *55*: 3003-3007, 1995.

5. Karnik, P., Plummer, S., Casey, G., Myles, J., Tubbs, R., Crowe, J. and Williams, B.R.G. Microsatellite instability at a single locus (D11S988) on chromosome 11p15.5 as a late event in mammary tumorigenesis. Human Mol Genet *4*: 1889-1894, 1995.

6. Takita, K-I., Sato, T., Miyagi, M., Watatani, M., Akiyama, F., Sakamoto, G., Kasumi, F., Abe, R. and Nakamura, Y. Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes. Cancer Res. *52*: 3914-3917, 1992.

7. Sait, S.N., Nowak, N.J., Singh-Kahlon, P., Weksberg, R., Squire, J., Shows, T.B., Higgins, M.J. Localization of Beckwith-Wiedemann and Rhabdoid tumor chromosome rearrangements to a defined interval in chromosome band 11p15.5. Genes Chromosomes Cancer *11*: 97-105, 1994.

8. Henry, I., Grandjouan, S., Couillin, P., Barichard, F., Huerre-Jeanpierre, C., Glaser, T., Philip, T., Lenoir, G., Chaussain, J.L., Junien, C. Tumor specific loss of 11p15.5 alleles in del 11p13 Wilms tumor and in familial adrenocortical carcinoma. Proc. Natl. Acad. Sci. *86*: 3247-3251, 1989.

9. Koufos, A., Hansen, M.F., Copeland, N.G., Jenkins, N.A., Lampkin, B.C. and Cavenee, W.K. Loss of heterozygosity in three embryonal tumours suggests a common pathogenetic mechanism. Nature (Lond) *316*: 330-334, 1985.

10. Sotel-Avila, D. and Gooch, W.M. III. Neoplasms associated with the Beckwith-Wiedemann Syndrome. Perspect. Pediatr. Pathol. *3*: 255-272. 1976.

11. Coppes, M.J., Bonetta, L., Huang, A., Hoban, P., Chilton-MacNeill,S., Campbell, C.E., Weksberg, R., Yeger, H., Reeve, A.E. and Williams, B.R.G. Loss of heterozygosity mapping in Wilms tumor indicates the involvement of three distinct regions and a limited role for nondisjunction or mitotic recombination. Genes, Chromosomes & Cancer *5*: 326-334, 1992.

12. Fearon, E.R., Feinberg, A.P., Hamilton, S.H. and Vogelstein, B. Loss of genes on the short arm of chromosome 11 in bladder cancer. Nature *318*: 377-380, 1985.

13. Viel, A., Giannini, F., Tumiotto, L., Sopracordevole, F., Visetin, M.C. and Biocchi, M. Chromosomal localisation of two putative 11p oncosuppressor genes involved in human ovarian tumours. Br. J. Cancer *66*: 1030-1036, 1992.

14. Bepler, G. and Garcia-Blanco, M.A. Three tumor-suppressor regions on chromosome 11p identified by high-resolution deletion mapping in human non-small cell lung cancer. Proc. Natl. Acad. Sci. USA *91*: 5513-5517, 1994.

15. Lothe, R.A., Fossa, S.D., Stenwig, A.E., Nakamura, Y., White, R., Borresen, A.L., Brogger, A. Loss of 3p or 11p alleles is associated with testicular cancer tumors. Genomics *5*: 134-138, 1989.

16. Wang, H.P. and Rogler, C.E. Deletions in human chromosome arms 11p and 13q in primary hepatocellular carcinomas. Cytogenet.Cell Genet. *48*: 72-78, 1988.

17. Birch, J.M., Hartley, A.L., Marsden, H.B., Harris, M. and Swindell, R. Excess risk of breast cancer in the mothers of children with soft tissue sarcomas. Br. J. Cancer *49*: 325-331, 1984.

18. Phillips, K.K., Welch, D.R., Miele, M.E., Lee, J-H., Wei, L.L. and Weissman, B.E. Suppression of MDA-MB-435 breast carcinoma cell metastasis following the introduction of human chromosome 11. Cancer Res. *56*: 1222-1227, 1996.

19. Karnik, P., Paris, M., Williams, BRG., Casey, G., Crowe, J., and Chen, P. Two distinct tumor suppressor loci within chromosome 11p15 implicated in breast cancer progression and metastasis. Human Mol. Gen. *7* 895-903, 1998.

20. Karnik, P., Chen, P., Paris, M., Yeger, H and Williams, BRG. Loss of heterozygosity at chromosome 11p15 in Wilms tumors: identification of two independent regions. Oncogene *17*, 237-24-, 1998.

21. Uberbacher,E.C. and R.J.Mural. Locating protein-coding regions in human DNA sequences by a multiple sensor-neural network approach. Proc. Natl.Acad.Sci. USA *88*: 11261-11265. 1991

22. Zhang, J. and T.L.Madden. PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation. Genome Res. *7*: 649-656. 1997

23. Schuler, et. al. A gene map of the human genome. Science *274*: 540-546. 1996

24. Neyfakh,A.A., V.E.Bidenko, and L.B.Chen. Efflux-mediated multidrug resistance in *Bacillus subtilis*:similarities and dissimilarities with the mammalian system. Proc. Natl. Acad. Sci. USA. *88*: 4781-4785, 1991.

25. Allard, J.D. and K.P. Bertrand. Sequence of a class E tetracycline resistance gene from *Escherichia coli* and comparison of related tetracycline efflux proteins. J. Bacteriol. *175*: 4554-4560, 1993.

26. Kyte, J. and R.F.Doolittle. A simple method for displaying the hydropathic character of a protein. J.Mol.Biol. *157*: 105-132, 1982.

27. Senapathy,P., M.B.Shapiro, and N.L.Harris. Splice junctions, branch point sites, and exons: Sequence statistics, identification, and applications to genome project. Methods Enzymol. *183*: 252-278, 1990.

28. Orita,M., H.Iwahana, H.Kanazawa, K.Hayashi, and T.Sekiya. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc.Natl.Acad.Sci. USA. *86*: 2766-2770. 1989

29. Ioannou, PA., Amemiya, CT., Garnes, J., Kraisel, PM., Shizuya, H., Chen, C., Bolzer, MA., and de Jong, P.J.. A new bacteriophage-derived vector for the propagation of large human DNA fragments. Nature Genet. *6*: 84-89. 1994

30. Tissot, C., and Mechti, N.. Molecular cloning of a new interferon-induced factor that represses human immunodeficiency virus type 1 long terminal repeat expression. J. Biol. Chem. *270*: 14891-8. 1995.

31. Hannigan, G.E., Bayani, J., Weksberg, R., Beatty, B., Pandita, A., Dedhar, S. and Squire, J.. Mapping of the gene encoding the integrin-linked kinase, ILK, to human chromosome 11p15.5-p15.4 Genomics *42*: 177-179, 1997.

32. Scheer, E., Mattei, M-G., Jacq, X., Chambon, P and Tora, L.. Organization and chromosomal localization of the gene (TAF2H) encoding the human TBP-associated factorII 30 ($TAF_{II}30$). Genomics *29*: 269-272, 1995.

33. Sambrook,J., E.F.Fritsch, and T.Maniatis. *Molecular Cloning: A laboratory manual,* 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 1989

34. Karnik,P., S.Plummer, G.Casey, J.Myles, R.Tubbs, J.Crowe. and BRG. Williams. Microsatellite instability at a single locus (D11S988) on chromosome 11p15.5 as a late event in mammary tumorigenesis Human Mol. Genet., *4*: 1889-1894, 1995.

Figure Legends:

Figure 1.

Scheme of the transcription map of LOH region 1 between the markers D11S1318 - D11S4088. Location of seven genes H19, IGF2, INS, TH, ASCL2, TAPA1 and KVLQT1 are shown. Novel unigene clusters (solid boxes) and ESTs (open boxes) and their expression data are shown.

Figure 2.

Representative ESTs from the unigene clusters hybridized to Multiple Tissue Northern Blots (Clontech) using standard techniques

Figure 3.

The *HET* gene. (A) Map location of polymorphic markers and some characterized genes in the chromosome 11p15.5 imprinted region. (B) Sequence of the human *HET* cDNA and predicted amino acidsequence. Start and stop codons and the polyadenylation signal are underlined.

Figure 4.

Hydrophobicity pattern of the deduced aminoacid sequence of *HET* according to the algorithm of Kyte and Doolittle, 1982. The horizontalscale shows the amino acid numbers. Regions (indicated positive) containing the maximally hydrophobic amino acid residues are numbered I-X.

Figure 5.

Northern blot analysis of mRNA from human tissues and cancer cell lines with *HET* cDNA probe. Constantly appearing hybridizing transcripts are approximately 4.4 kb and 1.5 kb; yet smaller transcripts are seen in some tissues. The cancer cell lines are promyelocytic leukemia (HL-60), HeLa cells (S3), chronic myelogenous leukemia (K562), lymphoblastic leukemia (MOLT-4), Burkitt lymphoma (Raji), colorectal adenocarcinoma (SW480), lung carcinoma (A549), and melanoma (G361).

Figure 6.

Exon-intron structure, and expression of the four isoforms of the *HET* gene.
A) Illustration of the four *HET* isoforms. Open boxes represent exons and lines indicate introns. Genomic DNA is displayed with the telomeric side on the left, and cDNA isoforms are displayed with the 5' end on the left. B) The nucleotide sequences of the novel exons 1a, 5a and 6a. Isoform 2 contains the same ORF as isoform 1. Exons 5a and 6a lack an initiation MET codon and introduce stop codons in the *HET* sequence. They may represent untranslated transcripts. C) Tissue specific expression of the *HET* isoforms 1, 2, 3 and 4. Multiple human tissues were analyzed using RT-PCR with novel exon specific primers (see methods).

Figure 7.

A. PCR strategy for assessing allele -specific expression of the human *HET* gene. Exons are boxed and the nucleotide polymorphisms are indicated by asterisks. The intron specific primers A6 and B6 were used to amplify the genomic DNA and the cDNA fragment harboring the polymorphism was amplified with the primers IP1 and IP2. Primer sequences are described in the text.

B. SSCA of exon 2 amplified (Primers A6 and B6) from matched normal (N) and tumor (T) DNAs from four Wilms tumor patients. Sample 1 is homozygous, samples 26T ,45T sand 35T show loss of heterozygosity. Arrows indicate the five variant alleles generated by the two polymorphisms.

C. Electropherograms of direct fluorescence sequencing of genomic DNA PCR products from samples 45N and 45T. The normal DNA shows A and G at the positions indicated, while the tumor DNA shows either an A or a G.

Figure 8.

RT-PCR analysis of expression of *HET* from fetal tissue samples. mRNA isolated from heterozygous fetal tissues were analyzed by RT-PCR as described in the text. Each set, A and B, includes PCR of genomic DNA isolated from fetal tissues, along with RT-PCRs of RNA from the indicated tissues.

Figure 9.

Graphic illustration of mapping results of LOH region 2 that spans the markers D11S1338-D11S1323. The map of chromosome 11p15 with the two LOH regions are shown. The Genomic contig across LOH region 2 and the ESTs and genes that mapped to this contig are indicated.

Figure 10.

Mapping of integrin -linked kinase (ILK), human TBP-associated factorII 30 (TAFII30) and a novel protease -like gene to the genomic contig of region 2 are shown. PCR reactions were performed withYAC847a12, YAC696H10, BAC1323 and PACILK as template DNA using primers specifically designed for these genes. Positive results are seen with YAC847a12 and PACILK, but not with YAC696H10 and BAC1323. These results enabled the mapping of these to LOH region 2 (shown in Fig. 9).

APPENDICES

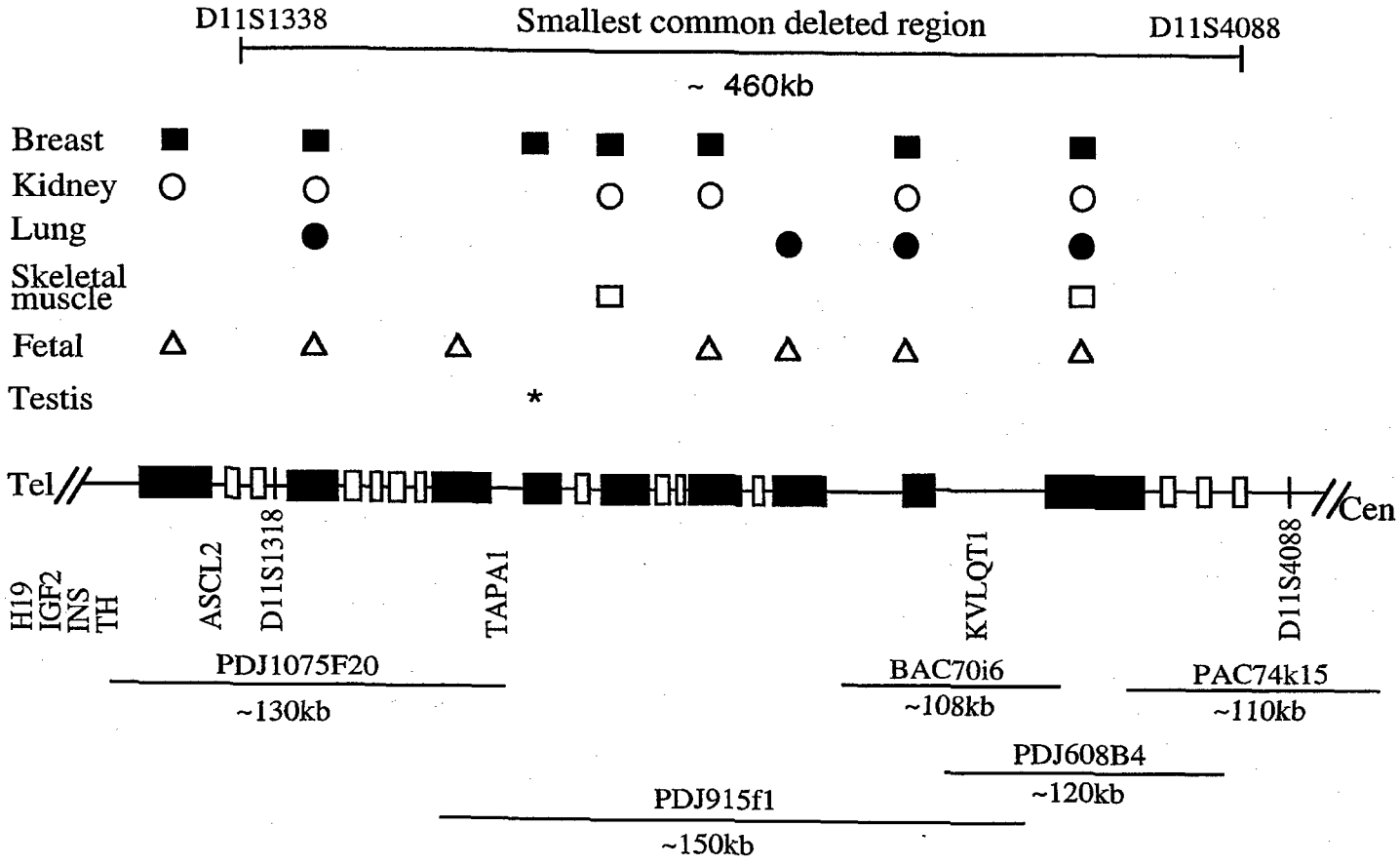


Fig 1. Scheme of the transcriptional map of the region between loci D11S1318 and D11S4088. Location of the seven genes H19, IGF2, INS, TH, ASCL2, TAPA1 and KVLQT1 are shown. The newly identified unigene clusters ▬ and transcripts ▭ and their expression data are indicated.
Specific expression: ■ in breast tissue, ○ in kidney, ● in lung, □ in skeletal muscle, △ in fetal tissue and * *in testis.*

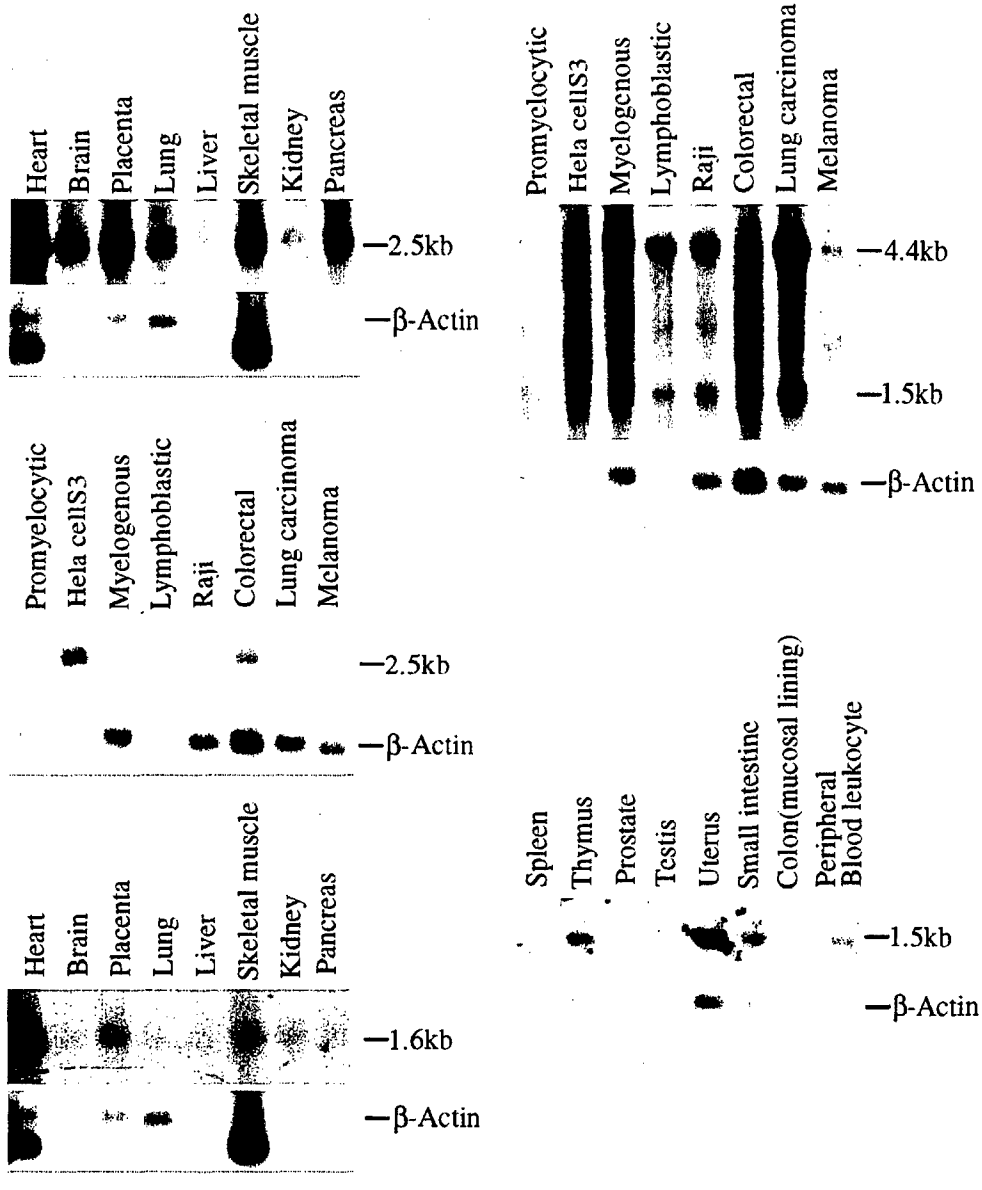
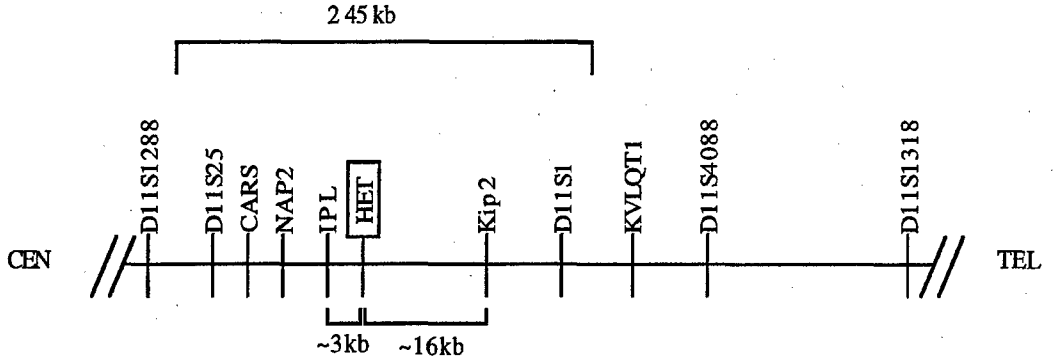


Fig 2. Northern blots of representative Unigene clusters.

A.



B.

aggtcaccctggagcgctcaccccaccggcaccagtgcccaagcccgcccctgcaaaggcaggcaaggccaggcgggtgctgcctgggacccagt
gactcagcacccctgcccggatcaactggacttttgccccctgctccgccagcctcctgcttggatctctcctgggtctccctgctgcgcctgtc
cagg

```
ATG CAG GGA GCT CGG GCT CCC AGG GAC CAG GGC CAG TCC CCC GGC AGG ATG AGC GCT CTA GGC CGG TCC TCG GTC ATC TTG CTT ACC TAC     90
M  Q  G  A  R  A  P  R  D  Q  G  Q  S  P  G  R  M  S  A  L  G  R  S  S  V  I  L  L  T  Y

GTG CTG GCC GCC ACA GAA CTT ACC TGC CTC TTC ATG CAG TTC TCC ATC GTG CCA TAC CTG TCT CGG AAA CTG GGC CTG GAT TCC ATT GCC    180
V  L  A  A  T  E  L  T  C  L  F  M  Q  F  S  I  V  P  Y  L  S  R  K  L  G  L  D  S  I  A

TTC GGC TAC CTG CAA ACC ACC TTC GGG GTG CTG CAG CTG CTG GGC GGG CCG GTG TTT GGC AGG TTC GCA GAC CAG CGC GGG GCG CGG GCG    270
F  G  Y  L  Q  T  T  F  G  V  L  Q  L  L  G  G  P  V  F  G  R  F  A  D  Q  R  G  A  R  A

GCG CTC ACG CTC TCC TTC CTG GCT GCC TTG GCG CTC TAC CTG CTC CTG GCG GCC GCC TCC AGC CCG GCC CTG CCC GGG GTC TAC CTG CTC    360
A  L  T  L  S  F  L  A  A  L  A  L  Y  L  L  L  A  A  A  S  S  P  A  L  P  G  V  Y  L  L

TTC GCC TCG CGC CTG CCC GGA GCG CTC ATG CAC ACG CTG CCA GCC GCC CAG ATG GTC ATC ACG GAC CTG TCG GCA CCC GAG GAG CGG CCC    450
F  A  S  R  L  P  G  A  L  M  H  T  L  P  A  A  Q  M  V  I  T  D  L  S  A  P  E  E  R  P

GCG GCC CTG GGC CGG CTG GGC CTC TGC TTC GGC GTC GGA GTC ATC CTC GGC TCC CTG CTG GGC GGG ACC CTG GTC TCC GCG TAC GGG ATT    540
A  A  L  G  R  L  G  L  C  F  G  V  G  V  I  L  G  S  L  L  G  G  T  L  V  S  A  Y  G  I

CAG TGC CCG GCC ATC CTG GCT GCC CTG GCC ACC CTC CTG GGA GCT GTC CTC AGC TTC ACC TGC ATC CCC GCC AGC ACC AAA GGG GCC AAA    630
Q  C  P  A  I  L  A  A  L  A  T  L  L  G  A  V  L  S  F  T  C  I  P  A  S  T  K  G  A  K

ACT GAC GCC CAG GCT CCA CTG CCA GGC GGC CCC CGG GCC AGT GTG TTC GAA CTG AAA GGC ATC GCC TCC CTG CTG CGG CTG CCA GAC GTC    720
T  D  A  Q  A  P  L  P  G  G  P  R  A  S  V  F  E  L  K  G  I  A  S  L  L  R  L  P  D  V

CCG AAG ATC TTC TTG GTG AAA GTG GCC TCC AAC TGC CCC ACA GGG CTC TTC ATG GTC ATG TTC TCC ATC ATC TCC ATG GAC TTC TTC CAA    810
P  K  I  F  L  V  K  V  A  S  N  C  P  T  G  L  F  M  V  M  F  S  I  I  S  M  D  F  F  Q

CTG GAG GCC GCC AAA GCT GGC TAC CTC ATG TCC TTC TTC GGG CTC CTC CAG ATG GTG ACC CAG GGC CTG GTC ATC GGG CAG CTG AGC AGC    900
L  E  A  A  K  A  G  Y  L  M  S  F  F  G  L  L  Q  M  V  T  Q  G  L  V  I  G  Q  L  S  S

CAC TTC TCG GAA GAG GTG CTG CTC GGG GCA AGC GTG CTG GTC TTC ATC GTG GTG GGC CTG GCC ATG GCC TGG ATG TCC AGC GTC TTC CAC    990
H  F  S  E  E  V  L  L  G  A  S  V  L  V  F  I  V  V  G  L  A  M  A  W  M  S  S  V  F  H

TTC TGC CTC CTG GTG CCC GGC CTG GTG TTC AGC CTC TGC ACC CTC AAC GTG GTC ACC GAC AGC ATG CTG ATC AAG GCT GTC TCC ACC TCG   1080
F  C  L  L  V  P  G  L  V  F  S  L  C  T  L  N  V  V  T  D  S  M  L  I  K  A  V  S  T  S

GAC ACA GGG ACC ATG CTG GGC CTC TGC GCC TCT GTA CAA CCA CTG CTC CGA ACT CTG GGA CCC ACG GTC GGC GGC CTC CTG TAC CGC AGC   1170
D  T  G  T  M  L  G  L  C  A  S  V  Q  P  L  L  R  T  L  G  P  T  V  G  G  L  L  Y  R  S

TTT GGC GTC CCC GTC TTC GGC CAC GTG CAG GTT GCT ATC AAT ACC CTT GTC CTC CTG GTC CTC TGG AGG AAA CCT ATG CCC CAG AGG AAG   1260
F  G  V  P  V  F  G  H  V  Q  V  A  I  N  T  L  V  L  L  V  L  W  R  K  P  M  P  Q  R  K

GAC AAA GTC CGG TGA CCG CTG CCC AGA CAC AGA CTG GCA ATA AAC TCC TAC TAA ATC CCA AAA AAA AAA AAA AAA A                  1336
D  K  V  R  *
```

Fig 3.

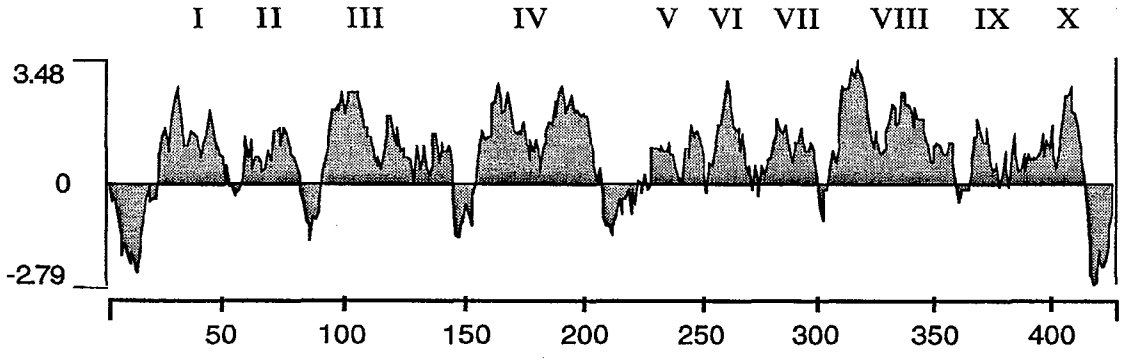

I
II
III
IV
V
VI
VII
VIII
IX
X
3.48
0
-2.79
50
100
150
200
250
300
350
400

Fig 4.

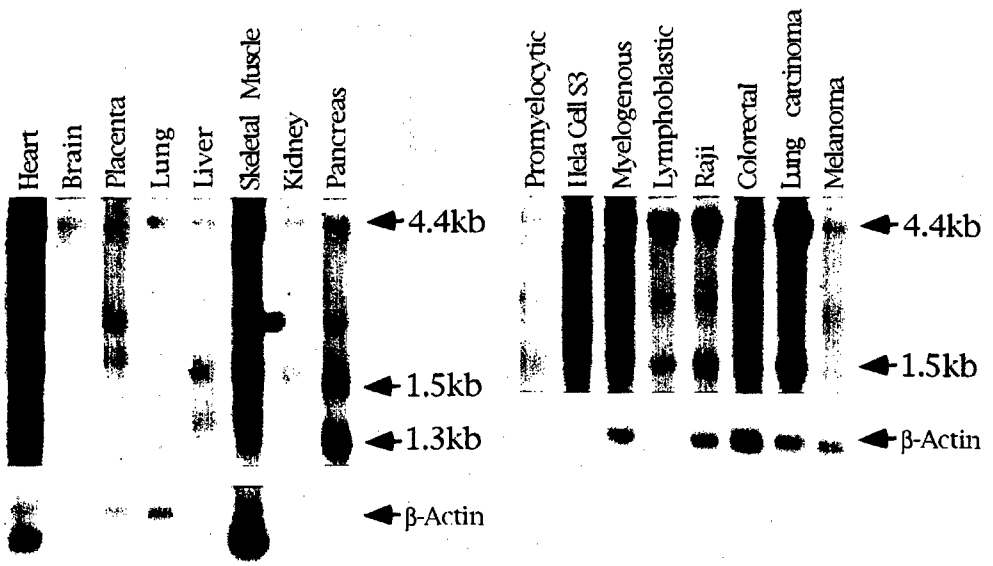
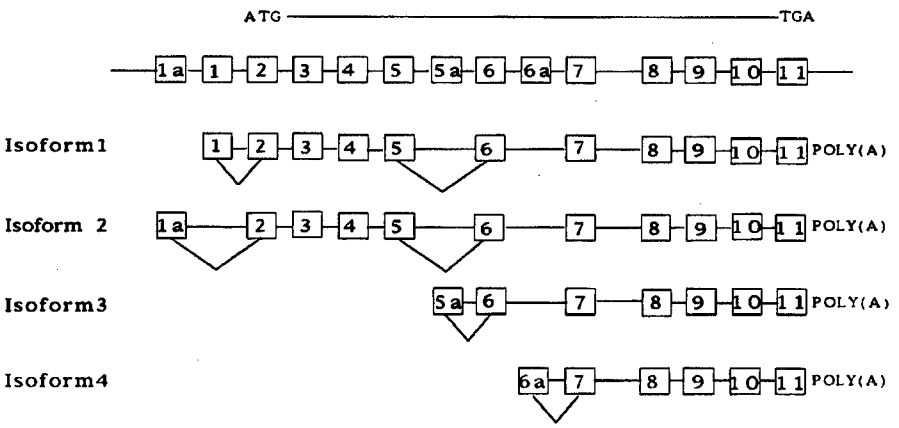

Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
4.4kb
1.5kb
1.3kb
β-Actin
Promyelocytic
Hela Cell S3
Myelogenous
Lymphoblastic
Raji
Colorectal
Lung carcinoma
Melanoma
4.4kb
1.5kb
β-Actin

Fig 5.

A.



B.

Exon 1a:

aggtctctccgatttcctctgctcaaccagttccctggctacctgaggccctgcttcacctggaggaagacagtggt

Exon5a:

gcaggcctctgaggccagggtaccaaagaaagggcccgtctctctggggctggccacaggggccagttgtccctcct
gcgccatacatctgtgcctgctggctggagggacaagctgtggcgcccgagcccagccgttccccttccctggcatt
ttcccaaaggaagggcccattcgtgcggaaccccagcagccagaaaacctgagtagctgacgtcctgggcggtgggt
gctgcaggagtcggagctgccctgatggattggcgggggcagcagcacaggtggggtggatatgccagcacccccct
agactgatgtgagcttaggggtcccacctcagaaatgaggggtggccaatgcccagactctctggactcagaggtgg
ccccactgactatcctagtcagtgtggctccaagtcccctccttgctctggcaaacggatgcaggt

Exon 6a:

caggggggcccctaggaatgcgtagaagtgtgctgaggggcggtgcccaacctggcagctggaagcatcaccagctcc
cattcccacaagt.

C.

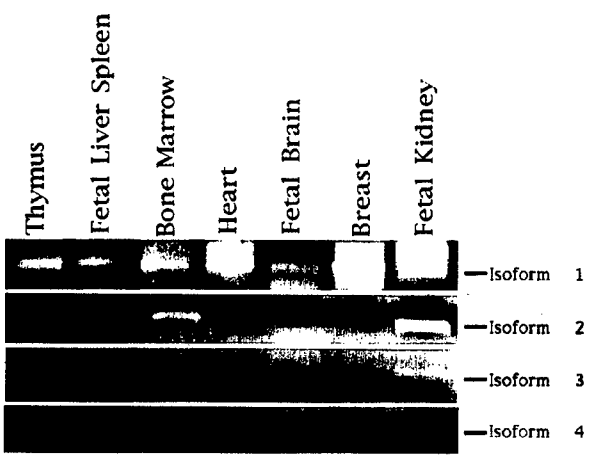
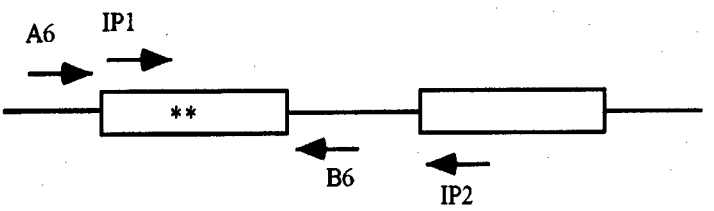
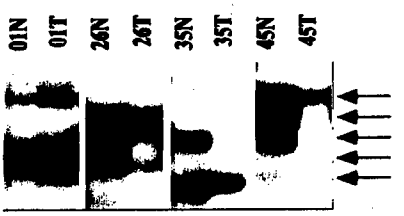


Fig 6.

A.


A6
IP1
**
B6
IP2

B.


01N
01T
26N
26T
35N
35T
45N
45T

C.

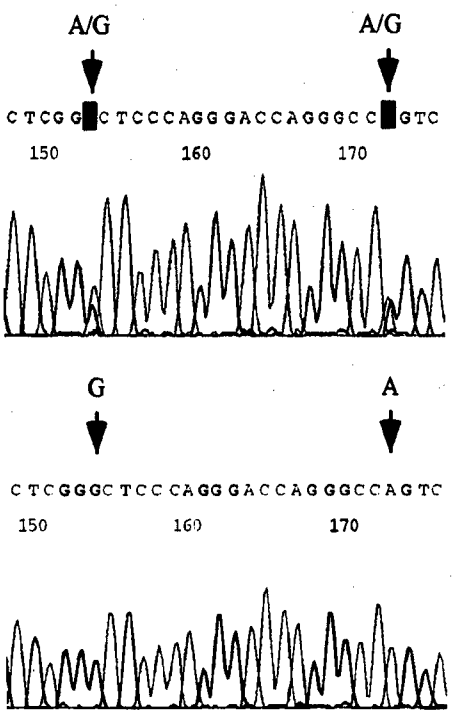

A/G
A/G
CTCGG C TCCCAGGGACCAGGGCC GTC
150
160
170
G
A
CTCGGGC TCCCAGGGACCAGGGCCAGTC
150
160
170

Fig 7.

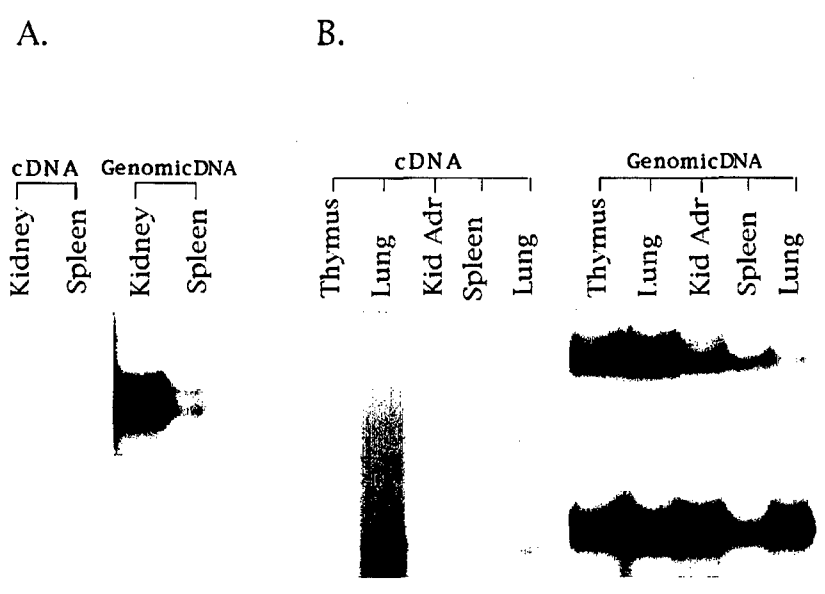

A.
B.
cDNA
GenomicDNA
Kidney
Spleen
Kidney
Spleen
cDNA
GenomicDNA
Thymus
Lung
Kid Adr
Spleen
Lung
Thymus
Lung
Kid Adr
Spleen
Lung

Fig 8.

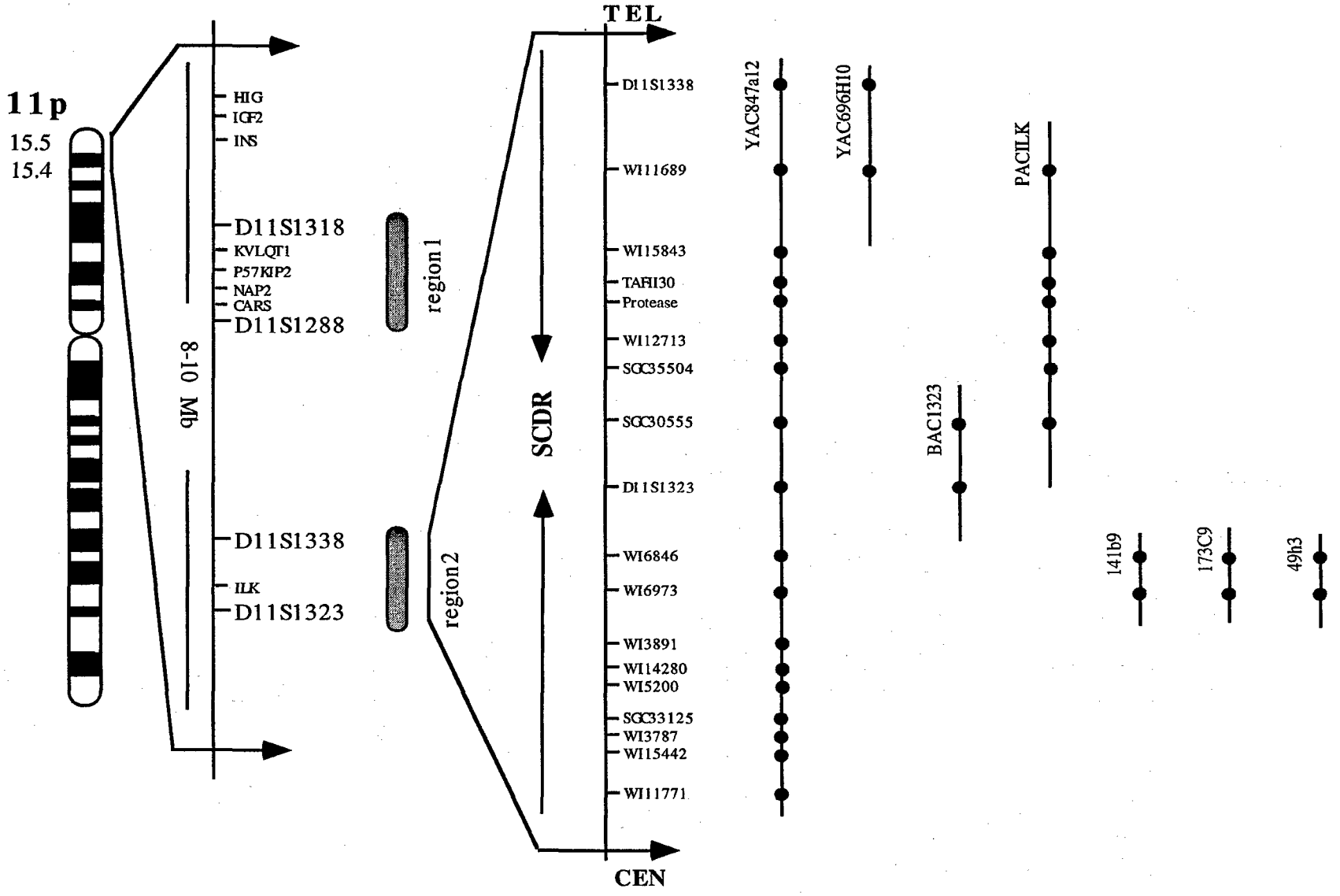

11p
15.5
15.4
HIG
IGF2
INS
D11S1318
KVLQT1
P57KIP2
NAP2
CARS
D11S1288
8-10 Mb
D11S1338
ILK
D11S1323
region 1
region 2
TEL
SCDR
CEN
D11S1338
WI11689
WI15843
TAHI30
Protease
WI12713
SGC35504
SGC30555
D11S1323
WI6846
WI6973
WI3891
WI14280
WI5200
SGC33125
WI3787
WI15442
WI11771
YAC847a12
YAC696H10
BAC1323
PACILK
141b9
173C9
49h3

Fig 9.

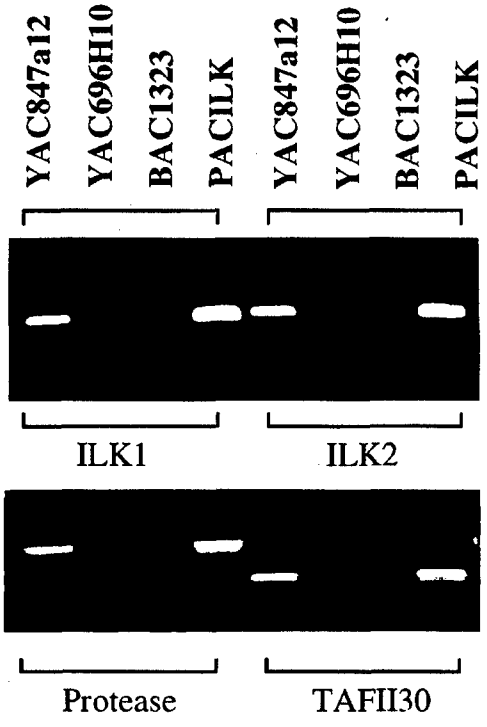


Fig 10. Mapping of ILK, TAFII30 and Protease genes on PACILK and YAC847a12. PCR reactions were performed on DNA templates of YAC847a12, YAC696H10, BAC1323 and PACILK using primers specifically designed for these genes. Positive results are seen in YAC847a12 and PACILK, but not in Yac696H10 and BAC1323.

Table 1

Splice Junction Sequences, Exon sizes, and
Estimated Intron Sizes of the HET Gene

Exon		Exon/Intron Junction		
#	Position	Donor Sequences	Acceptor Sequences	Intron Size (kb)
1	-194 to -132	GGCAGgtactc	ttgcagGCAAG	0.84
2	-131 to143	TGCCAgtgagt	tttcagTACCT	4.7
3	144 to 242	GGCAGgtacag	ccccagGTTCG	0.86
4	243 to 403	GCCAGgtaggt	ccccagCCGCC	0.22
5	404 to 536	TACGGgtgagt	ccgcagGATTC	6.908
6	537 to 655	GCCAGgtaagc	tgccagGCGGC	1.24
7	656 to 763	CACAGgtgagt	cggctcagGGCTC	1.20
8	764 to 863	CAGATGgtgagt	ctctagGTGAC	2.64
9	864 to 966	CCATGgtgagg	gtcccagGCCTG	0.23
10	967 to 1087	CACAGgtgagt	cccccagGGACC	2.39
11	1088 to 1336			

Table 2

Mapping of 18 ESTs in the LOH region 2 between the markers D11S1338 and D11S1323 on chromosome 11p15

ESTs	Gene Product	Samples analyzed						
		YAC 847a12	YAC 696H10	BAC 1323	Cosmid : 141b9	173c9	49h3	PAC ILK
SGC30555	60S ribosomal protein L21	+	-	+	-	-	-	+
SGC35504	Human staf50	+	-	-	-	-	-	+
WI12713	KIAA0409	+	-	-	-	-	-	+
WI3891		+	-	-	-	-	-	-
WI14280	Unidentified transcript	+	-	-	-	-	-	-
WI5200		+	-	-	-	-	-	-
SGC33125	50S ribosomal protein L17	+	-	-	-	-	-	-
WI3787		+	-	-	-	-	-	-
WI15442	Unidentified transcript	+	-	-	-	-	-	-
WI11771	Unidentified transcript	+	-	-	-	-	-	-
WI6846	Unidentified transcript	+	-	-	+	+	+	-
WI6973	SMPD1	+	-	-	+	+	+	-
D11S1338		+	+	-	-	-		-
D11S1323		+	-	+	-	-		-
WI11689	Unidentified transcript	+	+	-				+
WI15843	Unidentified transcript	+	-	-				+
TAFII30	TATA binding protein	+	-	-				+
Protease	Protease	+	-	-				+

Human Molecular Genetics, 1998, Vol. 7, No. 5 895–903

Two distinct tumor suppressor loci within chromosome 11p15 implicated in breast cancer progression and metastasis

Pratima Karnik[1,*], Mark Paris[1,2], Bryan R. G. Williams[1,2], Graham Casey[1], Joseph Crowe[3] and Ping Chen[1]

[1]Department of Cancer Biology, Lerner Research Institute and [3]Department of General Surgery and Breast Center, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA and [2]Department of Genetics, Case Western Reserve University, 2106 Adelbert Road, BRB 731, Cleveland, OH 44106, USA

Received January 9, 1998; Accepted February 6, 1998

Chromosome 11p15 has attracted considerable attention because of the biological importance of this region to human disease. Apart from being an important tumor suppressor locus showing loss of heterozygosity (LOH) in several adult and childhood cancers, 11p15 has been shown by linkage analysis to harbor the gene(s) for the Beckwith–Wiedemann syndrome. Furthermore, the clustering of known imprinted genes in the 11p15.5 region suggests that the target gene may also be imprinted. However, positional cloning efforts to identify the target genes have been complicated by the large size (~10 Mb) and complexity of LOH at 11p15. Here, we have analyzed 94 matched normal and breast tumor samples using 17 polymorphic markers that map to 11p15.5–15.4. We have defined precisely the location of a breast tumor suppressor gene between the markers *D11S1318* and *D11S4088* (~500 kb) within 11p15.5. LOH at this region occurred in ~35–45% of breast tumors analyzed. In addition, we have fine-mapped a second, critical region of LOH, that spans the markers *D11S1338–D11S1323* (~336 kb) at 11p15.5–p15.4, that is lost in ~55–60% of breast tumors. There is a striking correlation between the loss of the two 11p loci and the clinical and histopathological features of breast tumors. LOH at region 1 correlated significantly ($P = 0.016$) with early events in malignancy and invasiveness. In contrast, the loss of the more proximal region 2, is highly predictive ($P = 0.012$) of aggressive metastatic disease. Thus, two distinct tumor suppressor loci on chromosome 11p15 may contribute to tumor progression and metastasis in breast cancer. The fine mapping of this intriguing chromosomal region should facilitate the cloning of the target genes and provide critical clues to understanding the mechanisms that contribute to the evolution of adult and childhood cancers.

INTRODUCTION

Breast cancer is both genetically and clinically a heterogeneous and progressive disease. The severity of disease may be determined by the accumulation of alterations in multiple genes that regulate cell growth and proliferation. The inactivation of tumor suppressor genes, by a two-hit mechanism involving mutations and loss of heterozygosity (LOH), appears to be a common event in the genetic evolution of breast carcinomas (1). Several chromosome arms, including 1p, 1q, 3p, 11p, 11q, 13q, 16q, 17p, 17q and 18q, have been reported to show moderate (20–40%) to high (>50%) frequencies of LOH in breast tumors (1). This implies that multiple tumor suppressor genes are likely to be involved in the development and progression of breast cancer.

Genetic alterations at the short arm of chromosome 11 are a frequent event in the etiology of cancer (2–17). Several childhood tumors demonstrate LOH for 11p, including rhabdomyosarcoma (7,8), adrenocortical carcinoma (9), hepatoblastoma (10), mesoblastic nephroma (11) and Wilms' tumors (WT) (12). Recurrent LOH at 11p is also observed in adult tumors including bladder (13), ovarian (14), lung carcinomas (15), testicular cancers (16), hepatocellular carcinomas (17) and breast carcinomas (2–6), suggesting the presence of one or more critical tumor suppressor gene(s) involved in several malignancies.

Birch *et al.* (18) have reported an increased risk of breast cancer among mothers of children with embryonal rhabdomyosarcoma, providing genetic evidence for the apparent high-risk association between these two tumor types. The familial association between breast cancer and rhabdomyosarcoma and the other childhood tumors may well be the consequence of alterations in chromosome 11p15. The ability of a tumor suppressor gene(s) on chromosome 11 to re-establish control of the malignant phenotype has been demonstrated by transfer of a normal human chromosome 11 to the breast cancer cell line MDA-MB-435 (19). However,

*To whom correspondence should be addressed. Tel: +1 216 445 6529; Fax: +1 216 445 6269; Email: karnikp@cesmtp.ccf.org

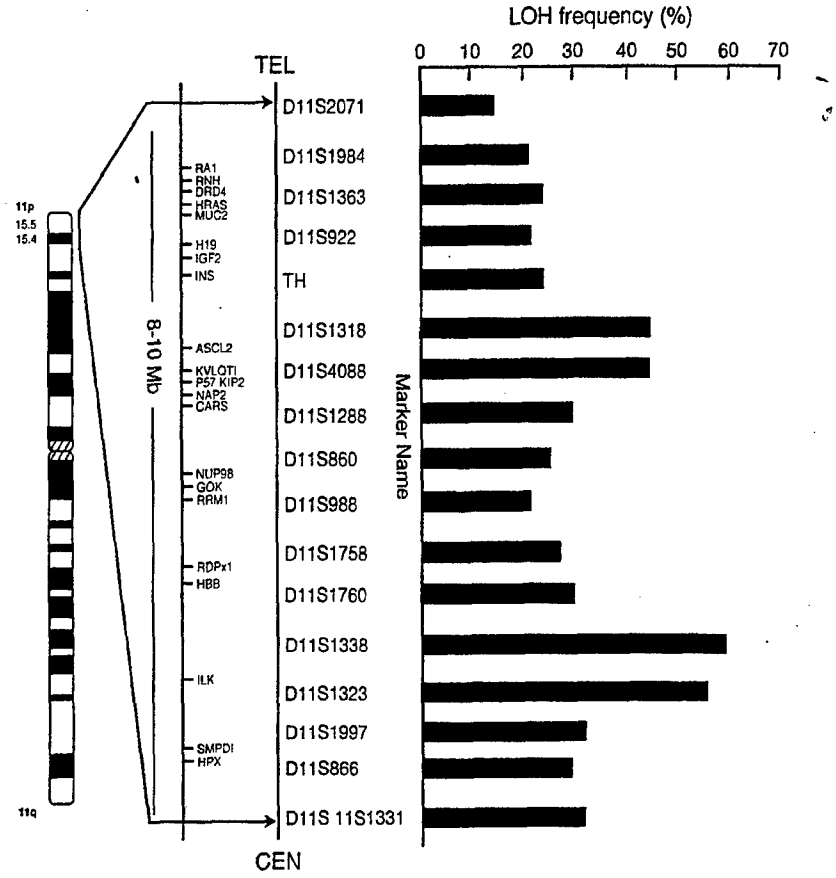


Figure 1. Representation of 11p15.5–15.4 and approximate location of the microsatellite repeats (21,22) and genes that map to this region [sequence map of chromosome 11 (http:// mcdermott.swmed.edu/)]. The histogram shows the percentage of LOH for each of the microsatellites in the informative breast tumor samples studied.

positional cloning efforts to identify the target genes on 11p15 have been complicated by the large size of this region (~10 Mb) and the complexity of LOH at 11p15.

With the goal of identifying the putative tumor suppressor gene(s) on chromosome 11p15, we have refined the minimal regions of LOH in this region, using a high-density marker analysis of 94 informative primary breast tumors and paired normal breast tissue. We have defined precisely and identified two distinct regions of chromosome 11p15.5–p15.4 that frequently are deleted in breast cancer. The association of LOH with clinical and histological parameters reveals the biological role of the putative tumor suppressor genes in the etiology of breast cancer.

RESULTS

Refinement of the tumor suppressor loci on chromosome 11p involved in breast cancer

Fluorescent PCR semi-automated genotyping (5) was used to detect and analyze allelic losses on chromosome 11 using a panel of 17 microsatellite markers. Previous studies have determined that this technique is more rapid and sensitive compared with the classical radioactive method in determining LOH in tumor DNAs (20). To identify the smallest common deleted region on chromosome 11p15 in breast tumors, 94 paired normal–tumor DNAs were assessed for LOH at 17 chromosome 11p15-specific microsatellite markers. These markers encompass the chromosomal sub-regions 11p15.5–11p15.4, estimated to be ~8–10 Mb (21,22) (Fig. 1). The results indicate that the loss of all or part of chromosome 11p is a more common event in human breast cancer than previously appreciated (3,4). LOH occurred in at least one marker on the short arm of chromosome 11 in 56 of 94 (60%) informative tumors. The overall frequency of LOH for each marker varies from 16 to 60%, with two peaks seen at markers *D11S1318* (45%) and *D11S1338* (60%) (Fig. 1). In addition to the 23% LOH at the *D11S988* locus (Fig. 1), there was a high incidence of microsatellite instability (MSI) at this marker as we had described earlier (5). Therefore, the possibility that MSI obscures the accurate determination of LOH at the *D11S988* locus in some of these tumors cannot be ruled out.

Tumors 57, 94, 6 and 24 (Genescans; Fig. 2) are illustrative examples of LOH patterns seen on chromosome 11p15 and provide a critical description of the LOH regions. Interstitial deletions, examples of which are seen in tumors 57, 94, 6 and 24, were observed more commonly than loss of the entire chromosomal arm as seen in tumor 7 (Fig. 3). In some cases, an example of which is seen at the marker *D11S1997* in tumor 24 (Fig. 2), it was observed that the peak for the allele which loses heterozygosity does not change between normal and tumor tissues. Rather, the peak for the other allele increases by several fold in the tumor.

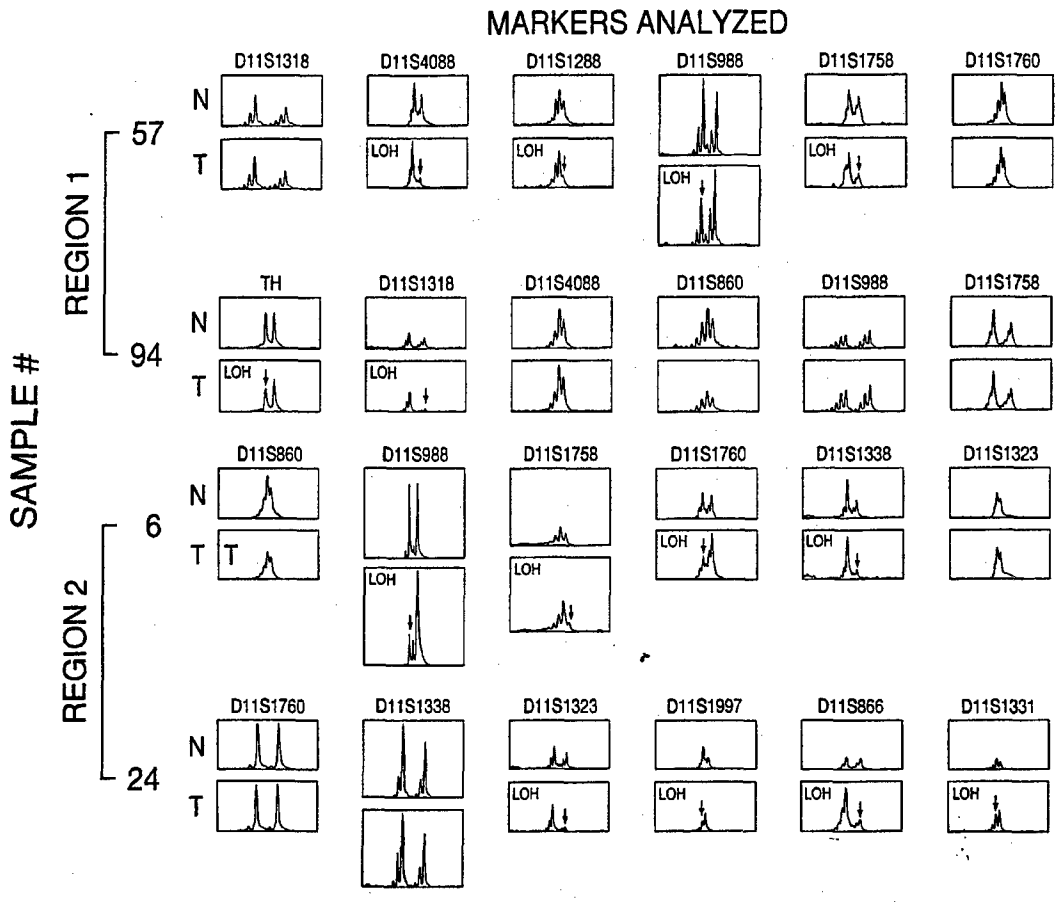


Figure 2. LOH studies of normal (N) and tumor (T) breast cancer pairs. Genescans of samples 57 (*D11S1318*, *D11S4088*, *D11S1288*, *D11S988*, *D11S1758* and *D11S1760*), 94 (*TH*, *D11S1318*, *D11S4088*, *D11S860*, *D11S988* and *D11S1758*), 6 (*D11S860*, *D11S988*, *D11S1758*, *D11S1760*, *D11S1338* and *D11S1323*) and 24 (*D11S1760*, *D11S1338*, *D11S1323*, *D11S1997*, *D11S866* and *D11S1331*) are shown. Arrows represent allelic loss. LOH represents samples that exhibit loss of heterozygosity and was calculated as described in the text.

Since the surrounding markers show LOH, we believe that this allelic imbalance represents LOH and not gene amplification.

The genotypes of the 13 representative breast tumors described in Figure 3, along with other tumors analyzed (data not shown), serve to refine and identify two distinct regions of LOH on 11p15. Region 1 is encompassed by markers *D11S1318* and *D11S4088* and is defined by the LOH break points in tumors 57 and 94. Tumor 57 retained heterozygosity for the markers *D11S2071*, *D11S1984*, *D11S1363*, *D11S922*, *TH* and *D11S1318*, but showed LOH for the markers *D11S4088*, *D11S1288*, *D11S860*, *D11S988* and *D11S1758*. This tumor also retained heterozygosity for all the remaining proximal markers. Tumor 94 showed LOH at markers *D11S2071*, *D11S1984*, *D11S922*, *TH* and *D11S1318*. This tumor was non-informative for the marker *D11S1363* and retained heterozygosity at all the proximal markers. Tumors 94 and 57, therefore, refine the LOH region 1 to a distance of ~500 kb between the markers *D11S1318* and *D11S4088*. This distance was calculated based on the estimation of Reid *et al.* (23) and the sequence map of chromosome 11 (http://mcdermott.swmed.edu/). Importantly, these results narrow the region containing this tumor suppressor gene from 2 Mb reported earlier (3,4) to ~500 kb. Tumors 42, 57 and 94 are examples of tumors that contain interstitial deletions exclusively in region 1 (Fig. 3).

The more centromeric region of LOH (region 2) is defined by breakpoints in the tumors 6 and 24 (Figures 2 and 3). Tumors 6, 24, 35, 45 and 76 are examples of tumors that harbor interstitial deletions in region 2 (Fig. 3). Tumor 6 showed LOH for the markers *D11S988*, *D11S1758*, *D11S1760* and *D11S1338*, but retained heterozygosity at all the markers distal to *D11S988* and at all the markers proximal to *D11S1338*. Tumor 24 was heterozygous for all the markers distal to *D11S1323* but showed LOH at *D11S1323*, *D11S1997*, *D11S866* and *D11S1331*. It is notable that tumors 6 and 24 exhibit LOH at either *D11S1338* or *D11S1323*, while the other locus retains heterozygosity. This clearly indicates that region 2 is within the interval that spans the markers *D11S1338–D11S1323*, a distance of ~336 kb based on the estimate of James *et al.* (22) and the sequence map of chromosome 11 (http://mcdermott.swmed.edu/). The yeast artificial chromosome (YAC) 847a12 that contains the markers *D11S1338*, *D11S1323* and *D11S1997* is ~1.4 Mb in length and is non-chimeric (STS-based map of the human genome; http:// www-genome.wi.mit.edu). We have identified integrin-linked kinase (*p59ILK*)

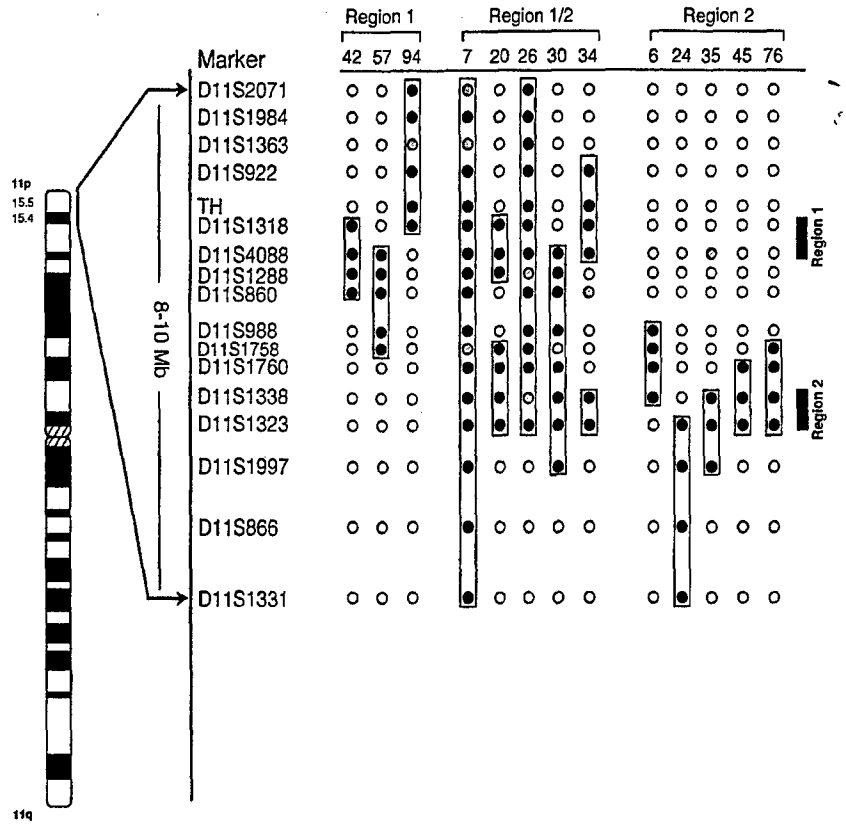


Figure 3. Genotypes of 13 representative tumors and the smallest regions of shared LOH in sporadic breast carcinoma. Tumor numbers are listed across the top, with the markers analyzed to the left. Open circles represent informative samples with no LOH; filled circles represent informative samples with LOH; and stippled circles represent non-informative (homozygous) samples. The maximum area of LOH is boxed for each LOH region in each tumor. The bars to the right represent the extent of the proposed common regions of LOH (regions 1 and 2). Tumors that exhibit LOH at region 1 only, regions 1 and 2, and region 2 only are grouped together.

as a candidate gene for this locus. *p59ILK* previously was mapped to the *CALC–HBBC* region on chromosome 11p15 (24). We have refined the map location of *p59ILK* and placed it on the YAC 847a12. PCR amplification of DNA from the YAC 847a12 with several different *p59ILK* primers produced the expected length fragments. No products were seen from a BAC DNA specific for the marker *D11S1323* or from yeast DNA (data not shown).

A total of five tumors, examples of which were seen in tumors 7, 20, 26, 30 and 34 (Fig. 3), appeared to have lost both of the regions on the chromosome 11p arm. In tumor 7 (Fig. 3), 14 of the 17 markers analyzed showed LOH. This tumor was non-informative for the markers *D11S2071*, *D11S1363* and *D11S1758*. The probability of three or more allelic losses in the same fragment being caused by independent events is small, and a series of LOH in contiguous markers is more likely to be due to deletion of the entire segment. In most instances, however, LOH on 11p15 appeared to be interstitial (e.g. tumors 20, 26, 30 and 34) and, therefore, restricted to relatively small chromosomal regions.

These data attest the presence of two distinct regions of LOH within 11p15.5–15.4. Region 1 lies between markers *D11S1318* and *D11S4088* (~500 kb) and region 2 lies between markers *D11S1338* and *D11S1323* (~336 kb). As described in Figure 3, the two regions were lost in different tumors, although in some tumors both of these regions appeared to be lost due either to interstitial deletions or to the loss of the entire 11p arm.

Negrini *et al.* (4) previously have reported a third LOH region, towards the telomere, between the markers *D11S576* and *D11S1318*. The percentage LOH that we observe for the telomeric markers *D11S2071*, *D11S1984*, *D11S1363* and *D11S922* (16–22%) is consistent with the observations of Negrini *et al.* (4). However, the percentage LOH for these markers is well within the background LOH seen at the remaining 11p markers (Fig. 1). In addition, we did not identify tumors that showed LOH exclusively in the telomeric markers *D11S2071–D11S922*. In our tumor panel, LOH at the distal markers occurred in concert with LOH at region 1. We therefore did not represent the distal region as an independent and third region of LOH.

Correlation between loss of heterozygosity at 11p and pathological features of breast tumors

Conflicting clinical data and clinical correlations of 11p LOH in breast cancer exist in the literature. This study was initiated with those concerns in mind. To examine the role of 11p LOH in breast cancer and to determine if the two regions are involved differentially in predicting the clinical course of this disease, we correlated our LOH data with the various clinical and histological parameters (Table 1).

Table 1. 11p LOH and clinico-pathological features of sporadic breast tumors

Clinical features	LOH in region 1		LOH in region 2		*P*-value
	N	%	*N*	%	
Ductal					
Yes	26	86.7	35	87.5	0.92
No	4	13.3	5	12.5	
If ductal					
In situ and invasive	4	15.4	0	0.0	0.016[a]
Invasive	22	84.6	35	100.0	
Lobular					
Yes	4	13.3	5	12.5	0.92
No	26	86.7	35	87.5	
If lobular					
In situ and invasive	1	25.0	0	0.0	0.24
Invasive	3	75.0	5	100.0	
Ploidy					
Diploid or near diploid	16	66.7	4	16.0	<0.001[a]
Aneuploid	8	33.3	21	84.0	
% S-phase cells					
≤10%	17	68.0	12	46.2	0.12
>10%	8	32.0	14	53.9	
ER/PR status					
ER+/PR+	3	23.1	8	50.0	0.23
ER+/PR–	7	53.9	4	25.0	
ER–/PR–	3	23.1	4	25.0	
Grade					
I–II	10	33.3	9	26.5	0.16
II–III	15	50.0	12	35.3	
III	5	16.7	13	38.2	
Lymphatic invasion					
Yes	4	28.6	20	69.0	0.012[a]
No	10	71.4	9	31.0	

[a]Statistically significant ($P < 0.05$).

All tumors described in Table 1 were infiltrating ductal carcinomas, which account for the largest single category of mammary carcinomas. The histological classification of the tumors, described under clinical features, was based on the WHO classification (25). Tumors were subdivided into two categories: (i) tumors that had lost only region 1 and (ii) tumors that had lost only region 2. Clinical features of breast tumors are summarized as frequencies and percentages, separately for each region. The χ^2 test was used to compare these features between regions 1 and 2. All statistical tests were performed using a 5% level of significance.

A correlation was observed between LOH in region 1 and breast tumors containing ductal carcinoma *in situ* (DCIS) synchronous with invasive carcinoma. Fifteen percent (4/26) of ductal tumors with LOH in region 1 contained breast cancer tissues with synchronous DCIS and invasive carcinoma, while none of the tumors with LOH in region 2 contained a DCIS component ($P = 0.016$). DCIS of the breast is considered a pre-invasive stage of breast cancer and may be a precursor of infiltrating breast cancer (26). Although the number of tumors analyzed is small, the statistically significant association between LOH in region 1 and such tumors suggests the involvement of a target gene in this region with early events in malignancy or invasiveness. The statistical analysis showed a significant association between 11p LOH and tumor ploidy. The majority of tumors (16/24) with region 1 LOH were either diploid or near diploid ($P < 0.001$). In contrast, the majority of tumors with region 2 LOH, were aneuploid ($P < 0.001$).

A trend was also observed between LOH at 11p and S-phase fraction (SPF). Fifty four percent of tumors with LOH in region 2, had a high SPF (>10% of cells in S-phase), compared with only 32% tumors with LOH in region 1. However, due to the small number of tumors in each category, statistical significance could not be established. It has been suggested that abnormal ploidy or elevated SPF identifies patients with shorter survival, and worsened disease-free survival, as well as being associated with poor outcome in locoregional control of the disease (27). The association between LOH at region 2 and tumors with high SPF and abnormal ploidy, that we observe, is therefore very relevant.

A striking correlation was observed between loss of region 2 and lymphatic invasion. Importantly, 69% of patients with 11p LOH in region 2 showed lymphatic invasion, whereas this infiltration was present in only 29% of patients with region 1 LOH. Thus, tumors that had lost region 2 reveal a significantly higher incidence of metastasis to a regional lymph node(s) ($P = 0.012$) than tumors that had lost region 1. Tumors that had lost the entire 11p arm, or had lost both regions, showed the clinicopathological features of tumors that had lost region 2. We also observed the trend that LOH in region 2 occurs more frequently in higher grade (grade III) tumors than LOH in region 1. Thus, LOH at region 2 may be a late event in mammary tumorigenesis, potentially enabling a clone of previously transformed cells to exhibit greater biological aggressiveness.

DISCUSSION

We have identified two distinct regions on chromosome 11p15 that are subject to LOH during breast tumor progression and metastasis. The high frequency of somatic loss of genetic information and the striking clinical correlation observed suggest their role in the pathogenesis of breast cancer.

We have defined precisely and narrowed the location of the putative tumor suppressor gene in region 1 from ~2 Mb (3,4) to ~500 kb. The critical region appears to extend between the markers *D11S1318* and *D11S4088* at 11p15.5. Previous studies (3,4) had only been able to place the putative gene in the larger overlapping area between *TH* and *D11S988* (Fig. 4). Although LOH frequencies for this region are consistent (24–45%, this report; 35%, ref. 3 and 22%, ref. 4), the peak incidence of LOH in this report is highest at *D11S1318*, ~1 Mb distal to the peak at *D11S860* reported by Winquist *et al.* (3) and Negrini *et al.* (4). This discrepancy may reflect the characteristics of the tumor samples analyzed or a difference in interpretation of the corresponding allelic patterns. LOH involving region 1 coincides with regions implicated in the pathogenesis of rhabdomyosarcoma (7,8), WT (7), ovarian carcinoma (14), stomach adenocarcinoma (28) and with a region conferring tumor suppressor activity

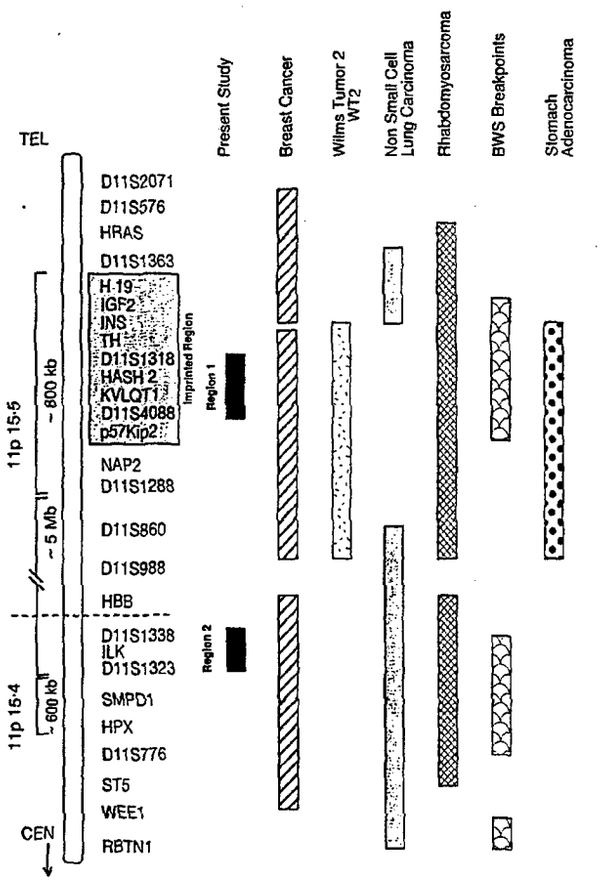


Figure 4. Schematic representation of regions on chromosome 11p15.5–15.4 harboring potential tumor suppressor and/or disease loci described in the present study and by other groups in breast cancer (2–5), Wilms tumor (7), non-small cell lung carcinoma (43), rhabdomyosarcoma (7), Beckwith–Wiedemann syndrome (31) and in stomach adenocarcinoma (28).

previously identified by genetic complementation experiments (29). Reid *et al.* (30) have used a functional assay to localize a 11p15.5 tumor suppressor gene that maps to this region in the G401 cell line. It is interesting to note that the physical map and contig of the *BWSCR1–WT2* region described recently (23) overlaps with our LOH region 1. Inversions and translocations at chromosome band 11p15.5, associated with malignant rhabdoid tumors and Beckwith–Wiedemann syndrome (31), also overlap with both regions of LOH in this study. It remains to be determined whether a single pleiotropic gene or a cluster of tumor suppressor genes play a role in the genesis of different cancers, possibly at different stages of tumor development and progression.

In childhood tumors such as WT and embryonal rhabdomyosarcoma, there is a strong bias toward loss of maternal 11p15 markers (32), suggesting the existence of an imprinted tumor suppressor gene in region 1. Since LOH for 11p15 is a common event in several adult tumors, a similar bias in allele loss could also be expected in the latter. Although the existence of parental bias towards LOH of 11p15 markers has not yet been demonstrated in adult tumors, two genes that map to 11p15, namely human insulin-like growth factor II gene (*IGF2*) and *H19*, are known to be expressed monoallelically in adult tissues (33,34), suggesting that genomic imprinting may be maintained in adult tissues. As illustrated in Figure 4, several genes that map to the LOH region 1 are subject to imprinting. It has been suggested that deregulation of imprinting may play a role during tumorigenesis (35,36). One model proposes that inappropriate methylation (hypermethylation) silences one copy of a tumor suppressor gene (36). This could be due to inappropriate activation of, or mutations in imprintor genes (37). If the first 'hit' represents the non-expression of one of the alleles due to the imprinting process, the second 'hit' may be mutational or may result from loss of all or part of the chromosome carrying the remaining functional tumor suppressor allele, thereby fulfilling Knudson's 'two-hit' hypothesis (38). Hypermethylation as an alternative pathway for tumor suppressor gene inactivation has been demonstrated elegantly for the retinoblastoma (*Rb1*) (39), the von Hippel Landau (*VHL*) syndrome (40), and the p16 tumor suppressor genes (37).

The other mechanism of altered imprinting that may affect tumorigenesis involves a gene activation hypothesis (36) that results in the reactivation of the silent allele due to the relaxation or loss of imprinting (LOI). LOI mutations have been detected at *H19*, *IGF2*, and to a lesser extent at p57KIP2 in *WT* and *BWS* (35). LOI of *IGF2* has been observed in benign and malignant breast tumors, in other adult and childhood tumors, and in a subgroup of patients with BWS (35,41). IGF2 is a potent mitogen with autocrine and paracrine effects, and the biallelic expression and possible increased dosage of the growth factor may explain the somatic overgrowth characteristics found in BWS patients and may play a role in tumor development. Although *IGF2* maps outside the LOH region 1, the presence of novel genes that map to region 1 that may have similar mitogenic effects cannot be ruled out. p57KIP2 (42) is a potential tumor suppressor candidate gene that maps to region 1. However, single strand conformation analysis and direct sequencing of 20 breast tumors (with LOH) and 15 breast tumors (without LOH) failed to reveal mutations in the coding region of this gene (P. Karnik *et al.*, unpublished observations).

The second hot-spot of LOH (region 2) in breast tumors is defined by markers *D11S1338–D11S1323*, and spans a distance of ~336 kb (22). Region 2 is centromeric to the putative *WT2* gene (region 1) and overlaps with LOH regions previously described for breast cancer (2) and non-small cell lung carcinoma (43). Importantly, we have refined this region from 5–10 Mb described earlier (2,43) to ~336 kb, with the highest incidence of LOH at the marker *D11S1338*. Previous studies (2,43), have only analyzed a few markers, sparsely distributed in the region proximal to *HBB*. Our study, therefore, is the first report of a detailed analysis of markers proximal to *HBB* that has considerably refined the boundaries of LOH in region 2.

We observed a significant correlation between LOH at the two chromosomal regions and the clinical and pathological parameters of the breast tumors. LOH in region 1 correlated with tumors that contain ductal carcinoma *in situ* synchronous with invasive carcinoma. This suggests that the loss of a critical gene in this region may be responsible for early events in malignancy or invasiveness. LOH at region 2 correlated with clinical parameters which portend a more aggressive tumor and a more ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. The association between 11p LOH, tumor progression and metastasis that we describe is analogous to the observations made

in other epithelial tumors including breast cancer (3,6). For example, LOH at 11p correlated with advanced T stage and nodal involvement in non-small cell lung carcinoma (44) as well as subclonal progression, hepatic involvement (45) and poor survival in ovarian and breast carcinomas (3,46). Phillips *et al.* (19) have shown that micro-cell-mediated transfer of a normal human chromosome 11 into the highly metastatic breast cancer cell line MDA-MB-435 had no effect on tumorigenecity in nude mice, but suppressed metastasis to the lung and regional lymph nodes. This further supports the observation that chromosome 11 harbors a metastasis suppressor gene. The integrin-linked kinase gene (24) has been shown to induce anchorage-independent growth and a tumorigenic phenotype in rodents. We have refined the map location of *p59ILK*, and placed this gene on the YAC 847a12 that spans the markers *D11S1338* and *D11S1323*. Thus, *p59ILK* is a tumor suppressor candidate for region 2.

It is not clear if LOH involving regions 1 and 2 act independently or synergistically in breast tumors. The identification of two subsets of tumors that have lost either region 1 or region 2 suggests that LOH at the two regions occurs independently and perhaps at different time points during breast tumor progression. This is consistent with the possibility that at least two tumor suppressor genes involved in the progression of breast cancer are located on the chromosome 11p15.5–15.4. These genes may function at distinct stages in the development and progression of breast cancer; alternatively, different target genes may be inactivated in different tumors. It is possible that specific subsets of tumors are defined by the particular set of mutations that they contain, which results in the clinical heterogeneity that is frequently seen in breast cancer.

Chromosome 11p15 contains several imprinted genes and two or more tumor suppressor genes. The fine mapping of this intriguing chromosomal region should facilitate the identification of novel genes, the evaluation of candidate genes and the establishment of the mechanisms whereby they contribute to the evolution of adult and childhood cancers.

MATERIALS AND METHODS

Patient materials and preparation of genomic DNA

Primary tumor and adjacent normal breast tissue samples were obtained from 94 randomly selected breast cancer patients undergoing mastectomy at the Cleveland Clinic Foundation (CCF). Samples of these tumors and corresponding non-involved tissue from each patient were collected at the time of surgery, snap-frozen and transferred to –80°C. Clinical and histopathological features of the tumors described in Table 1 were performed by the Pathology Department at CCF and were revealed only after the LOH study had been completed. The breast tumors described in Table 1 were classified according to the WHO classification (25). Tumor grading described in Table 1 was based on the Scarff–Bloom–Richardson method (25). DNA ploidy and S-phase determinations were done using the fine needle aspiration method (5). ER and PR status were done using the Ventana 320 automated immunostandard and the modified labeled streptavidin biotin technique (5).

An initial cryostat section was stained with H&E stain to determine the proportion of contaminating normal tissue, and only DNA purified from specimens thought to be highly enriched in tumor tissue was used for PCR. Generally we use tumor samples that contain <40% contamination of normal cells. In cases where LOH is questionable, where possible, regions containing a high proportion of normal tissue were physically removed from the original block by microdissection followed by DNA isolation. These improvements combined with the automatic quantitation of results using the Genescan Analysis have given us a better indication of LOH in tumor samples. Genomic DNA was isolated from normal and tumor tissue samples as described earlier (5) and quantitated by determining the optical densities at 260 and 280 nm.

Microsatellite polymorphisms and primers

DNA sequences flanking polymorphic microsatellite loci on chromosome 11p15.5 were obtained from the chromosome 11 databases and the Genome Data Base (GDB). Dye-labeled (FAM or HEX; Applied Biosystems) primers were either obtained from Research Genetics (Huntsville, AL) or synthesized as described earlier (5). Only one primer in each pair was fluorescently labeled so that only one DNA strand was detected on the gel. The physical distances between the polymorphic loci were calculated based on the sequence map of chromosome 11 (http://mcdermott.swmed.edu/) and the radiation hybrid map of James *et al.* (22). According to their calculation, 1 CR_{9000} = 50.2 kb.

Polymerase chain reaction (PCR) and analysis of PCR products using Genescan software

PCR of the DNA sequences was performed as described (5). PCR products were analyzed on Seaquate 6% DNA sequencing gels (Garvin, OK) in 1× TBE buffer in a Model 373A automated fluorescent DNA sequencer (Applied Biosystems) which is a four-color detection system. One µl of each PCR reaction was combined with 4 µl of formamide and 0.5 µl of a fluorescent size marker (ROX 350; Applied Biosystems). The gel was run for 6 h at 30 W. During electrophoresis, the fluorescence detected in the laser scanning region was collected and stored using the Genescan Collection software (Applied Biosystems). The fluorescent gel data collected during the run were analyzed automatically by the Genescan Analysis program (Applied Biosystems) at the end of each run. Each fluorescent peak was quantitated in terms of size (in base pairs), peak height and peak area.

LOH analysis with Genescan

Fluorescent technology (5) was used to detect and analyze CA repeat sequences. The ratio of alleles was calculated for each normal and tumor sample and then the tumor ratio was divided by the normal ratio, i.e. T1:T2/N1:N2, where T1 and N1 are the area values of the shorter length allele and T2 and N2 are area values of the longer allele product peak for tumor and normal respectively. We assigned a ratio of 0.70 or less to be indicative of LOH on the basis that tumors containing no normal contaminating cells and showing complete allele loss would theoretically give a ratio of 0.0, but because some tumors in this series contained an estimated 30–40% normal stromal cells (interspersed among the tumor cells), complete allele loss in these tumors would give an allele ratio of only 0.70. At least three independent sets of results were used to confirm LOH in each tumor.

Statistical analysis

Clinical features of breast tumors are summarized as frequencies and percentages, separately for each LOH region. The χ^2 test was

used to compare these features between regions 1 and 2. All statistical tests were performed using a 5% level of significance.

ACKNOWLEDGEMENTS

We wish to thank Dr John Cowell for the critical evaluation of the manuscript. This work was supported by grants from the US Army Medical Research and Materiel Command under DAMD17–96–1–6052 and the American Cancer Society, Ohio Chapter to P.K., and by the Betsey De Windt Cancer Research Fund.

REFERENCES

1. Callahan, R. and Campbell, G.N. (1989) Mutations in human breast cancer: an overview. *J. Natl Cancer Inst.*, **81**, 1780–1786.
2. Ali, I.U., Lidereau, R., Theillet, C. and Callahan, R. (1987) Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. *Science*, **238**, 185–188.
3. Winquist, R., Mannermaa, A., Alavaikko, M., Blanco, G., Taskinen, P.J., Kiviniemi, H., Newsham, I. and Cavenee, W. (1995) Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. *Cancer Res.*, **55**, 2660–2664.
4. Negrini, M., Rasio, D., Hampton, G.M., Sabbioni, S., Rattan, S., Carter, S.L., Rosenberg, A.L., Schwartz, G.F., Shiloh, Y., Cavenee, W.K. and Croce, C.(1995) Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3. *Cancer Res.*, **55**, 3003–3007.
5. Karnik, P., Plummer, S., Casey, G., Myles, J., Tubbs, R., Crowe, J. and Williams, B.R.G. (1995) Microsatellite instability at a single locus (*D11S988*) on chromosome 11p15.5 as a late event in mammary tumorigenesis. *Hum. Mol. Genet.*, **4**, 1889–1894.
6. Takita, K.-I., Sato, T., Miyagi, M., Watatani, M., Akiyama, F., Sakamoto, G., Kasumi, F., Abe, R. and Nakamura, Y. (1992) Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes. *Cancer Res.*, **52**, 3914–3917.
7. Besnard-Guerin, C., Newsham, I., Winquist, R. and Cavenee, W.K. (1996) A common loss of heterozygosity in Wilms tumor and embryonal rhabdomyosarcoma distal to the *D11S988* locus on chromosome 11p15.5. *Hum. Genet.*, **97**, 163–170.
8. Visser, M., Sijmons, C., Bras, J., Arceci, R.J., Godfried, M., Valentijn, L.J., Voute, P.A. and Baas, F. (1997) Allelotype of pediatric rhabdomyosarcoma *Oncogene*, **15**, 1309–1314.
9. Henry, I., Grandjouan, S., Couillin, P., Barichard, F., Huerre-Jeanpierre, C., Glaser, T., Philip, T., Lenoir, G., Chaussain, J.L. and Junien, C. (1989) Tumor specific loss of 11p15.5 alleles in del 11p13 Wilms tumor and in familial adrenocortical carcinoma. *Proc. Natl Acad. Sci. USA*, **86**, 3247–3251.
10. Koufos, A., Hansen, M.F., Copeland, N.G., Jenkins, N.A., Lampkin, B.C. and Cavenee, W.K. (1985) Loss of heterozygosity in three embryonal tumours suggests a common pathogenetic mechanism. *Nature*, **316**, 330–334.
11. Sotel-Avila, D. and Gooch, W.M. III (1976) Neoplasms associated with the Beckwith–Wiedemann syndrome. *Perspect. Pediatr. Pathol.*, **3**, 255–272.
12. Coppes, M.J., Bonetta, L., Huang, A., Hoban, P., Chilton-MacNeill, S., Campbell, C.E., Weksberg, R., Yeger, H., Reeve, A.E. and Williams, B.R.G. (1992) Loss of heterozygosity mapping in Wilms tumor indicates the involvement of three distinct regions and a limited role for nondisjunction or mitotic recombination. *Genes Chromosomes Cancer*, **5**, 326–334.
13. Fearon, E.R., Feinberg, A.P., Hamilton, S.H. and Vogelstein, B. (1985) Loss of genes on the short arm of chromosome 11 in bladder cancer. *Nature*, **318**, 377–380.
14. Viel, A., Giannini, F., Tumiotto, L., Sopracordevole, F., Visetin, M.C. and Biocchi, M. (1992) Chromosomal localisation of two putative 11p onco-suppressor genes involved in human ovarian tumours. *Br. J. Cancer*, **66**, 1030–1036.
15. Bepler, G. and Garcia-Blanco, M.A. (1994) Three tumor-suppressor regions on chromosome 11p identified by high-resolution deletion mapping in human non-small cell lung cancer. *Proc. Natl Acad. Sci. USA*, **91**, 5513–5517.
16. Lothe, R.A., Fossa, S.D., Stenwig, A.E., Nakamura, Y., White, R., Borresen, A.L. and Brogger, A. (1989) Loss of 3p or 11p alleles is associated with testicular cancer tumors. *Genomics*, **5**, 134–138.
17. Wang, H.P. and Rogler, C.E. (1988) Deletions in human chromosome arms 11p and 13q in primary hepatocellular carcinomas. *Cytogenet. Cell Genet.*, **48**, 72–78.
18. Birch, J.M., Hartley, A.L., Marsden, H.B., Harris, M. and Swindell, R. (1984) Excess risk of breast cancer in the mothers of children with soft tissue sarcomas. *Br. J. Cancer*, **49**, 325–331.
19. Phillips, K.K., Welch, D.R., Miele, M.E., Lee, J.-H., Wei, L.L. and Weissman, B.E. (1996) Suppression of MDA-MB-435 breast carcinoma cell metastasis following the introduction of human chromosome 11. *Cancer Res.*, **56**, 1222–1227.
20. Schwengel, D.A., Jedlicka, A.E., Nanthakumar, E.J., Weber, J.L. and Levitt, R.C. (1994) Comparison of fluorescence-based semi-automated genotyping of multiple microsatellite loci with autoradiographic technique. *Genomics*, **22**, 46–54.
21. van Heyningen, V. and Little, P.F.R. (1995) Report of the Fourth International Workshop on Chromosome 11 Mapping. *Cytogenet. Cell Genet.*, **69**, 127–158.
22. James, M.R., Richard, C.W. III, Schott, J.J., Yousry, C., Clark, K., Bell, J., Terwilliger, J.D., Hazan, J., Dubay, C., Vignal, A., Agrapart, M., Imai, T., Nakamura, Y., Polymeropoulos, M., Weissenbach, J., Cox, D.R. and Lathrop, G.M. (1994) A radiation hybrid map of 506 STS markers spanning human chromosome 11. *Nature Genet.*, **8**, 70–76.
23. Reid, L.H., Davies, C., Cooper, P.R., Crider-Miller, S.J., Sait, S.N.J., Nowak, N.J., Evans, G., Stanbridge, E.J., de Jong, P., Shows, T.B., Weissman, B.E. and Higgins, M.J. (1997) A 1-Mb physical map and PAC contig of the imprinted domain in 11p15.5 that contains TAPA1 and the BWSCR1/WT2 region. *Genomics*, **43**, 366–375.
24. Hannigan, G.E., Bayani, J., Weksberg, R., Beatty, B., Pandita, A., Dedhar, S. and Squire, J. (1997) Mapping of the gene encoding the integrin-linked kinase, ILK, to human chromosome 11p15.5–p15.4. *Genomics*, **42**, 177–179.
25. Tavassoli, F.A. (1992) *Pathology of the Breast*. Appleton and Lange, Norwalk, CT.
26. Fujii, H., Marsh, C., Cairns, P., Sidransky, D. and Gabrielson, E. (1995) Genetic progression, histological grade, and allelic loss in ductal carcinoma *in situ* of the breast. *Cancer Res.*, **56**, 1493–1497.
27. Merkel, E. and McGuire, W.L. (1990) Ploidy, proliferative activity and prognosis. DNA flow-cytometry of solid tumors. *Cancer*, **65**, 1194–1205.
28. Baffa, R., Negrini, M., Mandes, B., Rugge, M., Ranzani, G.N., Hirohashi, S. and Croce, C.M. (1996) Loss of heterozygosity for chromosome 11 in adenocarcinoma of the stomach. *Cancer Res.*, **56**, 268–272.
29. Koi, M., Johnson, L.A., Kalikin, L.M., Little, P.F.R., Nakamura, Y. and Feinberg, A.P. (1993) Tumor cell growth arrest caused by subchromosomal transferable DNA fragments from chromosome 11. *Science*, **260**, 361–364.
30. Reid, L.H., West, A., Gioeli, D.G., Phillips, K.K., Kelleher, K.F., Araujo, D., Stanbridge, E.J., Dowdy, S.F., Gerhard, D.S. and Weissman, B.E. (1996) Localization of a tumor suppressor gene in 11p15.5 using the G401 Wilms tumor assay. *Hum. Mol. Genet.*, **5**, 239–247.
31. Hoovers, J.M.N., Kalikin, L.M., Johnson, L.A., Alders, M., Redeker, B., Law, D.J., Bliek, J., Steenman, M., Benedict, M., Wiegant, J., Lengauer, C., Taillon-Miller, P., Schlessinger, D., Edwards, M.C., Elledge, S.J., Ivens, A., Westerveld, A., Little, P., Mannens, M. and Feinberg, A.P. (1995) Multiple genetic loci within 11p15 defined by Beckwith–Wiedemann syndrome rearrangement breakpoints and subchromosomal transferable fragments. *Proc. Natl Acad. Sci. USA*, **92**, 12456–12460.
32. Junien, C. (1992) Beckwith–Wiedemann syndrome, tumorigenesis and imprinting. *Curr. Opin. Genet. Dev.*, **2**, 431–438.
33. Tycko, B. (1994) Genomic imprinting: mechanism and role in human pathology. *Am. J. Pathol.*, **144**, 431–443.
34. Estratiadis, A. (1994) Parental imprinting of autosomal mammalian genes. *Curr. Opin. Genet. Dev.*, **4**, 265–280.
35. Reik, W. and Maher, E.R. (1997) Imprinting in clusters: lessons from Beckwith–Wiedemann syndrome. *Trends Genet.*, **13**, 330–334.
36. Feinberg, A.P. (1993) Genomic imprinting and gene activation in cancer. *Nature Genet.*, **4**, 110–113.
37. Little, M. and Wainwright, B. (1995) Methylation and *p16*: suppressing the suppressor. *Nature Med.*, **1**, 633–634.
38. Knudson, A.G. and Strong, L.C. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl Acad. Sci. USA*, **68**, 820–823.
39. Sakai, T., Herman, J.G., Latif, F., Weng, Y., Lerman, M.I., Zbar, B., Liu, S., Samid, D., Duan, D.S., Gnarra, J.R. and Linehan, W.M. (1991) Allele-specific hypermethylation of the retinoblastoma tumor suppressor gene. *Am. J. Hum. Genet.*, **48**, 880–888.
40. Herman, J.G., Sakai, T., Togauchida, J., Ohtani, N., Yandell, D.W., Rapaport, J.M. and Dryja, T.P. (1994) Silencing of the VHL tumor suppressor gene by DNA methylation in renal carcinoma. *Proc. Natl Acad. Sci. USA*, **91**, 9700–9704.

41. McCann, A.H., Miller, N., O'Meara, A., Pedersen, I., Keogh, K., Gorey, T. and Dervan, P.A. (1996) Biallelic expression of the *IGF2* gene in human breast disease. *Hum. Mol. Genet.*, **5**, 1123–1127.
42. Matsuoka, S.,Thomson, J.S., Edwards, M.C., Bartletta, J.M., Grundy, P., Kalikin, L.M., Harper, J.W., Elledge, S.J. and Feinberg, A.P. (1996) Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, p57KIP2, on chromosome 11p15. *Proc. Natl Acad. Sci. USA*, **93**, 3026–3030.
43. Tran, Y.K. and Newsham, I.F. (1996) High-density marker analysis of 11p15.5 in non-small cell lung carcinomas reveals allelic deletion of one shared and one distinct region when compared to breast carcinomas. *Cancer Res.*, **56**, 2916–2921.
44. Fong, K.M., Zimmerman, P.V. and Smith, P.J. (1994) Correlation of loss of heterozygosity at 11p with tumor progression and survival in non small cell lung cancer. *Genes Chromosomes Cancer*, **10**, 183–189.
45. Vandamme, B., Lissens, W., Amfo, K., De Sutter, P., Bourgain, C., Vamos, E. and De Greve, J. (1992) Deletion of chromosome 11p13–1p15.5 sequences in invasive human ovarian cancer is a subclonal progression factor. *Cancer Res.*, **52**, 6646–6652.
46. Eccles, D.M., Gruber, L., Stewart, M., Steel, C.M. and Leonard, R.C. (1992) Allele loss on chromosome 11p is associated with poor survival in ovarian cancer. *Dis. Markers*, **10**, 95–99.

http://www.stockton-press.co.uk/onc

Loss of heterozygosity at chromosome 11p15 in Wilms tumors: identification of two independent regions

Pratima Karnik[1], Ping Chen[1], Mark Paris[1], Herman Yeger[2] and Bryan RG Williams[1]

[1]*Department of Cancer Biology, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA; and* [2]*Hospital for Sick Children, Toronto, Ontario, Canada*

Loss of heterozygosity (LOH) on the short arm of chromosome 11 is the most frequent genetic alteration in Wilms tumors, indicating that one or more tumor suppressor genes that map to this chromosomal region are involved in the development of the disease. The WT1 gene located on 11p13 has been characterized but mutations in this gene occur in only about 10% of Wilms tumors. A second locus (WT2) at chromosome 11p15 has also been described in Wilms tumors but thus far efforts to clone the WT2 gene(s) have been frustrated by the large size (~10 Mb) of this region. Using a high-density marker LOH analysis of 11p15.5–15.4, we have refined the location of a Wilms tumor suppressor gene between the markers D11S1318–D11S1288 (~800 kb) within 11p15.5. We have also identified a second, novel region of LOH that spans the markers D11S1338–D11S1323 (~336 kb) at 11p15.5–p15.4. Thus a second distinct locus, in addition to the previously defined WT2, on chromosome 11p15.5, appears to play a role in the development of Wilms tumors.

Keywords: Wilms tumor; tumor suppressor genes; chromosome 11p15; loss of heterozygosity

Introduction

Wilms tumor (WT), a childhood kidney cancer which occurs in approximately 1 per 10 000 live births, is responsible for about 400 tumors per year in the USA (Young *et al.*, 1986). The majority of cases are diagnosed by 6 years of age, although rare cases have been documented in adolescents and adults. The inactivation of tumor-suppressor genes, by a two-hit mechanism involving mutations and loss of heterozygosity (LOH), appears to be a common event in the development of Wilms tumors (Knudson and Strong, 1972). Several chromosomal regions, including 1p, 11p13, 11p15 and 16q show consistent genetic changes in tumor tissue (Grundy *et al.*, 1995), suggesting that multiple suppressor genes are likely to be involved in the etiology of the disease. The association of Wilms tumor with aniridia, genito-urinary abnormalities and mental retardation (the WAGR syndrome), and cytogenetically visible alterations of chromosome 11p13 led to the identification of the WT1 locus (Franke *et al.*, 1979). The characterization of homozygous deletions observed in individuals with WAGR and in tumors from sporadic WT patients enabled the isolation of the WT1 gene (Bonetta *et al.*, 1990; Huang *et al.*, 1990; Call *et al.*, 1990; Gessler *et al.*, 1990). Analyses of WT1 mutations in patients with Denys-Drash syndrome (intersex disorders and nephropathy coupled with Wilms tumor), established the WT1 gene as an important tumor suppressor which also played a crucial role in urogenital development (Pelletier *et al.*, 1991). However, extensive mutational analyses of WT1 in sporadic Wilms tumors indicates that mutations occur at a frequency of only about 10% (Varanasi *et al.*, 1994). A second locus on chromosome 11p15 (WT2) shows a high frequency of loss of heterozygosity in Wilms tumors (Coppes *et al.*, 1992). Furthermore, linkage analysis and cytogenetic rearrangements have mapped the gene(s) for Beckwith-Weidemann syndrome (BWS) to 11p15.5 (Ping *et al.*, 1989). BWS is characterized by hemihypertrophy, macroglossia, umbilical hernia, and an increased risk for developing childhood cancers including Wilms tumors (Sotelo-Avila and Gooch, 1976). Functional studies using chromosome mediated gene transfer into the rhabdoid tumor cell line G401 have also been used to map a tumor suppressor locus to 11p15 (Reid *et al.*, 1996). Since positional cloning efforts to identify the target genes on 11p15 are complicated by the large size of this region (~10 Mb) and complexity of LOH, we have refined the mapping of the WT2 locus by performing a detailed LOH analysis of the 11p15.5–11p15.4 region using a high-density marker analysis of 38 informative tumor DNAs. The earlier described WT2 locus, has been refined from 2 Mb (Besnard-Guerin *et al.*, 1996) to ~800 kb. We have also identified a second novel locus of approximately 336 kb, that is proximal to WT2 and is also frequently lost in Wilms tumors. Thus two distinct regions of LOH on chromosome 11p15, appear to play a role in the development of Wilms tumors.

Results

To identify the smallest common deleted region on chromosome 11p15 in Wilms tumors, thirty eight tumor DNAs and matching constitutional DNA samples were assessed for LOH at 15 chromosome 11p15-specific polymorphic loci. These markers encompass the chromosomal sub-regions 11p15.5–11p15.4, estimated to be ~8–10 Mb (James *et al.*, 1994). LOH occurred in at least one marker on the short arm of chromosome 11 in 16 of 38 (43%) informative tumors. The results confirm that interstitial deletions and the loss of all or part of chromosome 11p are very common events in Wilms tumors.

Correspondence: P Karnik
Received 10 November 1997; revised 9 March 1998; accepted 9 March 1998

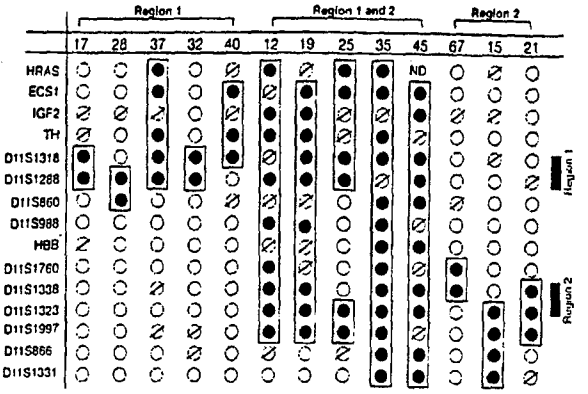


Figure 1 Genotypes of thirteen representative tumors and the smallest regions of shared LOH in Wilms tumors: Tumor numbers are listed across the top, with the markers analysed to the left. Open circles represent informative samples, with no LOH; filled circles represent informative samples with LOH; ND represents not determined and stippled circles represent non-informative (homozygous) samples. The maximum area of LOH is boxed for each LOH region in each tumor. The bars to the right represent the extent of the proposed common regions of LOH (region 1 and region 2). Tumors that exhibit LOH at region 1 only, region 1 and region 2, and region 2 only are grouped together

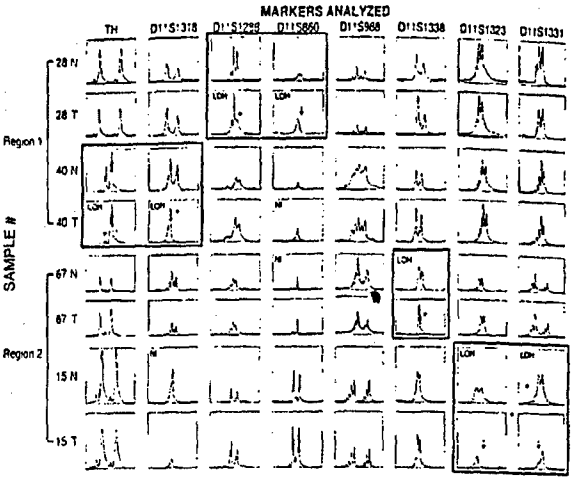


Figure 2 LOH studies of normal (N) and tumor (T) Wilms tumor pairs. Genescans of samples 28 and 40 (region 1) and samples 67 and 15 (region 2) for the markers TH, D11S1318, D11S1288, D11S860, D11S988, D11S1338, D11S1323 and D11S1331 are shown. Arrows represent allelic loss. NI- non informative for the marker. LOH represents samples that exhibit loss of heterozygosity. Samples showing LOH are boxed

The genotypes of the 13 representative Wilms tumors (Figure 1), along with other tumors analysed (data not shown), serve to identify and refine the LOH regions on 11p15. Figure 2 depicts the primary genescan data of the four critical tumors 28, 40, 67 and 15, showing the restricted areas of LOH. Interstitial deletions, examples of which were seen in tumors 17, 28, 37, 32, 40, 25, 67, 15 and 21, (Figure 1) define the LOH breakpoints and provide a critical description of the region. Two regions of LOH at 11p15 can be identified. The telomeric region (region 1) is encompassed by the markers TH and D11S1288 and is defined by the LOH breakpoints in tumors 28 and 40 as identified using Genescan (Figure 2). Tumor 28 retained heterozygosity for HRAS, ECS1, TH and D11S1318, but showed LOH for the markers D11S1288 and D11S860. ECS1, is a novel gene that we have recently mapped to the 11p15 region (Paris *et al.*, in preparation). Tumor 28 was non-informative for the marker IGF2 but retained heterozygosity for the proximal markers D11S988, HBB, D11S1760, D11S1338, D11S1323, D11S1997, D11S866 and D11S1331. Tumor 40 showed LOH at markers ECS1, TH and D11S1318 and was non-informative for the markers HRAS and IGF2 and for the proximal marker D11S860. This tumor was heterozygous for all the remaining markers including D11S1288. Tumors 28 and 40, therefore, refine the LOH Region 1 to a distance of ~800 kb between the markers D11S1318 and D11S1288. Importantly, these results narrow the region containing this tumor suppressor gene from 2 Mb reported earlier (Besnard-Guerin *et al.*, 1996) to ~800 kb.

The more centromeric region of LOH (region 2) is defined by breakpoints in the tumors 67 and 15 (Figures 1 and 2). Tumor 67 was heterozygous for the markers HRAS, ECS1, TH, D11S1318, D11S1288, D11S988 and HBB and non-informative for D11S860 and IGF2. This tumor showed LOH for the markers D11S1760 and D11S1338 and was heterozygous for the remaining proximal markers. Tumor 15 showed LOH at the markers D11S1323, D11S1997, D11S866 and D11S1331. This tumor was non-informative for the markers HRAS, IGF2, D11S1318 and was heterozygous at all the remaining markers. It is notable that tumors 67 and 15 exhibit LOH at either D11S1338 or D11S1323. This suggests that region 2 is within the interval that spans the markers D11S1338–D11S1323 a distance estimated to be ~336 kb (James *et al.*, 1994). We have mapped integrin-linked kinase (p59ILK) on the yeast artificial chromosome (YAC) 847a12 that harbors the markers D11S1338 and D11S1323. PCR amplification of the DNA from the YAC 847a12 with several different p59ILK primers produced the expected length fragments (data not shown). No amplification products were observed from a BAC DNA specific for the marker D11S1323 or from yeast DNA. Thus p59ILK is a candidate tumor suppressor gene for this locus. p59ILK was previously mapped to the CALC-HBBC region on chromosome 11p15 (Hannigan *et al.*, 1997).

The data described indicate the presence of two distinct regions of LOH within 11p15.5–15.4. Five of the tumors in the group studied, examples of which are 12, 19, 25, 35 and 45 (Figure 1), appeared to have lost both of the LOH regions on the chromosome 11p arm. However, only tumors 35 and 45 in this group appeared to have also lost the 11p13 markers (Coppes *et al.*, 1992) suggesting the loss of the entire 11p arm in these tumors.

Discussion

We have identified two distinct regions on chromosome 11p15 that are subject to LOH in Wilms tumors. The two LOH regions overlap with the LOH map that we have previously described for breast cancer (Karnik *et*

al., 1998). LOH involving region 1 coincides with regions implicated in the pathogenesis of rhabdomyosarcoma (Besnard-Guerin *et al.*, 1996; Sait *et al.*, 1994), breast cancer (Karnik *et al.*, 1998; Winquist *et al.*, 1995), ovarian carcinoma (Viel *et al.*, 1992), stomach adenocarcinoma (Baffa *et al.*, 1996) and with a region conferring tumor suppressor activity previously identified by genetic complementation experiments (Reid *et al.*, 1996; Koi *et al.*, 1993). Inversions and translocations at chromosome band 11p15.5, associated with Beckwith-Wiedemann syndrome and malignant rhabdoid tumors (Sait *et al.*, 1994; Hoovers *et al.*, 1995) overlap with both regions of LOH in this study. Importantly, we have more precisely refined the location of the putative tumor suppressor gene in region 1 from 2 Mb (Besnard-Guerin *et al.*, 1996) to 800 kb. As illustrated in Figure 3, several genes that map to this region are subject to allele-specific imprinting (Reid *et al.*, 1997) thereby raising the possibility that the tumor suppressor gene that maps to 11p15.5 may be imprinted in a tissue-specific manner. Given the size of LOH region 1, it is possible that a single pleiotropic gene rather than a cluster of genes may play a role in the genesis of different cancers, possibly at different stages of tumor development and progression. More extensive analysis and isolation of the target gene that maps to this region will be important to establish whether loss or alteration of the same or different genes is involved in each of these cases. p57KIP2 and NAP2 (Reid *et al.*, 1997) are potential tumor suppressor candidate genes that map to region 1. However, single strand conformation analysis and direct sequencing of Wilms tumors failed to reveal mutations in these genes (Karnik *et al.*, unpublished observations).

In addition to the previously described WT2 locus, we have identified a second region of LOH (region 2) at 11p15 in Wilms tumors. This novel LOH locus in Wilms tumors, is defined by markers D11S1338–D11S1323, which spans a distance of ~336 kb and is centromeric to the putative WT2 gene. We have narrowed this region from 5–10 Mb (Tran and Newsham, 1996; Ali *et al.*, 1987) to ~336 kb, with the highest incidence of LOH, at the marker D11S1338. Integrin-linked kinase (p59ILK) was earlier shown to map to the CALC-HBBC region and was shown to induce anchorage-independent growth and a tumorigenic phenotype in rodents (Hannigan *et al.*, 1997). We have refined the map location of p59ILK, and placed this gene on the yeast artificial chromosome (YAC) 847a12, that harbors the markers D11S1338 and D11S1323. Thus, p59ILK is a potential tumor suppressor candidate for region 2.

These findings suggest the presence of two distinct tumor suppressor loci on chromosome 11p15 that appear to play a role in the development of Wilms tumors. The fine-mapping of these loci should lead to the cloning of the target genes and the establishment of mechanisms that contribute to the development of Wilms tumor and other childhood and adult cancers.

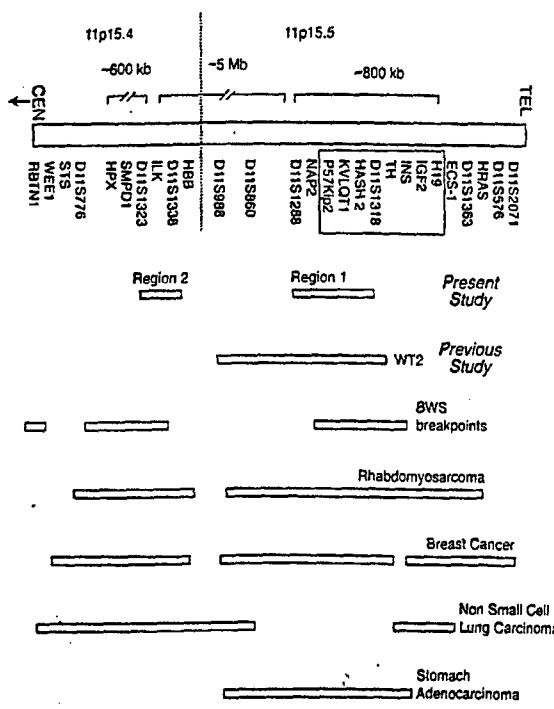


Figure 3 Schematic representation of regions on chromosome 11p15.5–15.4 harboring potential tumor suppressor and/or disease loci described in the present study and by other groups in Wilms tumor, Beckwith-Wiedemann syndrome, Rhabdomyosarcoma, breast cancer, non small cell lung carcinoma and stomach adenocarcinoma. Several imprinted genes that map to the WT2 region are boxed

Materials and methods

Patient materials and preparation of genomic DNA

Constitutional and tumor DNA from patients were obtained for 38 Wilms tumor cases as described previously (Coppes *et al.*, 1992). Thirty-three patients had unilateral Wilms tumors and five had bilateral disease. The median age at diagnosis was 3 years (range, 3 months to 14.7 years). There were 18 male and 20 female patients. Constitutional DNA was obtained from peripheral blood leukocytes, lymphoblastoid cell lines, primary skin fibroblast cultures, or normal kidney tissue. Tumor tissues taken for DNA and RNA analyses were directly snap-frozen in liquid nitrogen.

Microsatellite polymorphisms and primers

DNA sequences flanking polymorphic loci on chromosome 11p15.5 were obtained from the Genome Data Base (GDB). The sequence analysis and characterization of the new gene, ECS1 will be described elsewhere (Paris *et al.*, in preparation). Dye labeled (FAM or HEX from Applied Biosystems) primers were either obtained from Research Genetics (Huntsville, Alabama) or synthesized as described earlier (Karnik *et al.*, 1995). One primer in each pair was fluorescently labeled so that only one DNA strand was detected on the gel.

Polymerase Chain Reaction (PCR) and analysis of PCR products using genescan software

PCR of the DNA sequences was performed as described (Karnik *et al.*, 1995). PCR products were analysed on Seaquate 6% DNA sequencing gels (Garvin, OK) in 1×TBE buffer in a Model 373A automated fluorescent DNA sequencer (Applied Biosystems) which is a four color detection system. One ml of each PCR reaction was combined with 4 ml formamide and 0.5 ml of a fluores-

was run for 6 h at 30 W. During electrophoresis, the fluorescence detected in the laser scanning region was collected and stored using the Genescan Collection software (Applied Biosystems). The fluorescent gel data collected during the run was automatically analysed by the Genescan Analysis program (Applied Biosystems) at the end of each run. Each fluorescent peak was quantitated in terms of size (in base pairs), peak height and peak area.

LOH analysis with Genescan

To detect the presence and extent of LOH in the tumors, we analysed constitutional and tumor DNA from each patient for CA repeat sequences using fluorescent technology (Karnik *et al.*, 1995). In most Wilms tumors, LOH was complete loss of one allele. The ratio of alleles was calculated for each normal and tumor sample and then the tumor ratio was divided by the normal ratio, i.e. T1 : T2/N1 : N2, where T1 and N1 are the area values of the shorter length allele and T2 and N2 are area values of the longer allele product peak for tumor and normal respectively. At least three independent sets of results were used to confirm LOH in each tumor.

Acknowledgements

This work was supported by grants from the US Army Medical Research and Material Command under DAMD17-96-1-6052 and American Cancer Society, Ohio Chapter to PK; and by the NIDDK grant to BRGW.

References

Ali IU, Lidereau R, Theillet C and Callahan R. (1987). *Science*, **238**, 185–188.

Baffa R, Negrini M, Mandes B, Rugge M, Ranzani GN, Hirohashi S and Croce CM. (1996). *Cancer Res.*, **56**, 268–272.

Besnard-Guerin C, Newsham I, Winquist R and Cavenee WK. (1996). *Hum. Genet.*, **97**, 163–170.

Bonetta L, Kuehn SE, Huang A, Law DJ, Kalikin LM, Koi M, Reeve AE, Brownstein BH, Yeger H, Williams BRG and Feinberg AP. (1990). *Science*, **250**, 994–997.

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C and Housman DE. (1990). *Cell*, **60**, 509–520.

Coppes MJ, Bonetta L, Huang A, Hoban P, Chilton-MacNeill S, Campbell CE, Weksberg R, Yeger H, Reeve AE and Williams BRG. (1992). *Genes, Chromosomes and Cancer*, **5**, 326–334.

Franke U, Holmes LB, Atkins L and Riccardi VM. (1979). *Cytogenet. Cell Genet.*, **24**, 185–192.

Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH and Brunns GA. (1990). *Nature*, **343**, 774–778.

Grundy P, Coppes MJ and Haber D. (1995). *Hematology/Oncology Clinics Of North America*, **9**, 1201–1215.

Hannigan GE, Bayani J, Weksberg R, Beatty B, Pandita A, Dedhar S and Squire J. (1997). *Genomics*, **42**, 177–179.

Hoovers JMN, Kalikin LM, Johnson LA, Alders M, Redeker B, Law DJ, Bliek J, Steenman M, Benedict M, Wiegant J, Lengauer C, Taillon-Miller P, Schlessinger D, Edwards MC, Elledge SJ, Ivens A, Westerveld A, Little P, Mannens M and Feinberg AP. (1995). *Proc. Natl. Acad. Sci.*, **92**, 12456–12460.

Huang A, Campbell C, Bonetta L, McAndrews-Hill M, Chilton-MacNeil S, Coppes MJ, Law DJ, Feinberg AP, Yeger H and Williams BRG. (1990). *Science*, **250**, 991–994.

James MR, Richard CW III, Schott JJ, Yousry C, Clark K, Bell J, Terwilliger JD, Hazan J, Dubay C, Vignal A, Agrapart M, Imai T, Nakamura Y, Polymeropoulos M, Weissenbach J, Cox DR and Lathrop GM. (1994). *Nat. Genet.*, **8**, 70–76.

Karnik P, Paris M, Williams BRG, Casey G, Crowe J and Chen P. (1998). *Human Mol. Genet.*, (in press).

Karnik P, Plummer S, Casey G, Myles J, Tubbs R, Crowe J and Williams BRG. (1995). *Human Mol. Genet.*, **4**, 1889–1894.

Knudson AG and Strong LC. (1972). *J. Natl. Cancer Inst.*, **48**, 313–324.

Koi M, Johnson LA, Kalikin LM, Little PFR, Nakamura Y and Feinberg AP. (1993). *Science*, **260**, 361–364.

Pelletier J, Bruening W, Li FP, Haber DA, Glaser T and Housman DE. (1991). *Nature*, **353**, 431–434.

Ping AJ, Reeve AE, Law DJ, Young MR, Boehnke M and Feinberg AP. (1989). *Am. J. Hum. Gen.*, **44**, 720–723.

Reid LH, West A, Gioeli DG, Phillips KK, Kelleher KF, Araujo D, Stanbridge EJ, Dowdy SF, Gerhard DS and Weissman BE. (1996). *Hum. Mol. Genet.*, **5**, 239–247.

Reid LH, Davies C, Cooper PR, Crider-Miller SJ, Sait SNJ, Nowak NJ, Evans G, Stanbridge EJ, de Jong P, Shows TB, Weissman BE and Higgins MJ. (1997). *Genomics*, **43**, 366–375.

Sait SN, Nowak NJ, Singh-Kahlon P, Weksberg R, Squire J, Shows TB and Higgins MJ. (1994). *Genes Chromosomes Cancer*, **11**, 97–105.

Sotelo-Avila C and Gooch WM III. (1976). *Perspect. Pediatr. Pathol.*, **3**, 255–272.

Tran YK and Newsham IF. (1996). *Cancer Research*, **56**, 2916–2921.

Varanasi R, Bardeesy N, Ghahremani M, Petruzzi MJ, Nowak N, Adam MA, Grundy P, Shows TB and Pelletier J. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 3554–3558.

Viel A, Giannini F, Tumiotto L, Sopracordevole F, Visetin MC and Biocchi M. (1992). *Br. J. Cancer*, **66**, 1030–1036.

Winquist R, Hampton GM, Mannermaa A, Blanco G, Alavaikko M, Kiviniemi H, Taskinen PJ, Evans GA, Wright FA, Newsham I and Cavenee W. (1995). *Cancer Res.*, **55**, 2660–2664.

Young JLJ, Ries LG, Silverberg E, Horm JW and Miller RW. (1986). *Cancer*, **58**, 598–602.